A STUDY OF ALTERNARIOSIS,

A SEEDBORNE DISEASE

OF

ZINNIA ELEGANS JACQ.

CAUSED BY THE FUNGUS

ALTERNARIA ZINNIAE PAPE.

A Thesis Presented in Partial Fulfilment
of the Requirements for the Degree of
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INTRODUCTION.

The ornamental zinnia (*Zinnia elegans Jacq.*), is one of the most popular of the late summer and autumn flowering annuals. The species originated in Mexico as a simple-headed flower type, and was later introduced to North America and Europe where new types were quickly created offering great variation in plant form and shades of blooms. In New Zealand the zinnia is particularly popular, being in great demand as a bedding plant for use in home gardens and public parks. Seedlings are rsised commercially by nurserymen from seed imported mainly from Europe and North America.

There have been widespread reports throughout New Zealand in recent years of severe losses in zinnia sowings from a disease characterised by post-emergence damping off due to stem lesioning at or above ground level, and severe spotting of foliage. Nursery conditions created by dense sowing, and the maintenance of high humidity within boxes by overhead watering seem to be conducive to rapid build-up and spread of the disease. Attacks have frequently been so severe as to render complete sowings unsaleable.

The only foliage disease of zinnias recorded in New Zealand is Alternaria leaf spot caused by the fungus *ALTERNARIA ZINNIAE PAPE* (Dingley and Brien 1956). In this report the authors describe symptoms on the foliage and stems of adult plants, and state some of the morphological and physiological properties of the casual organism. No mention is made of symptoms having been found in this country on seedlings, although they state the disease has been reported overseas as being seedborne, and causing a seedling blight as well as leaf spot symptoms on adult plants.

Disease outbreaks in seedling zinnis sowings have been frequently experienced by New Zealand nurserymen using sterilised soil, in seed imported from countries where Alternaria leafspot is well established. This suggested the possibility of such outbreaks being, in fact, a seedling phase of Alternaria leaf spot, the source of primary inoculum being the seed. In preliminary experimental work isolations from infected cotyledons and seedlings showing post-emergence damping off due to stem lesioning, consistently yielded a species c

Alternaria identical in all respects to Alternaria zinniae isolated from foliage lesions of adult plants. Mature plants inoculated with Alternaria isolates from naturally diseased seedlings produced leafspot symptoms identical with those commonly observed in field plantings of zinnias, and caused by Alternaria zinniae. Observations and preliminary experimentation thus warranted the hypothesis that in New Zealand there is one Alternaria species pathogenic to zinnias and capable of causing infection of both seedlings and adult plants, the primary infection arising from the use of imported seed carrying viable inoculum of the fungus.

Alternaria zinniae was first reported from Denmark in 1902 by Rostrup (Baker and Davis 1950), and has since been recorded from many other countries. In spite of the wide distribution of the disease and frequent reports of severe losses there have been very few detailed studies of the disease and casual organism. The most notable of these are papers by Pape (1942), Dimock and Osborn (1943), Neergaard (1945), and Baker and Davis (1950). Moreover, a study of these papers reveals major gaps in knowledge of the pathogen, and also several conflicting statements concerning the mode of secondary spread of the fungus, the method(s) by which the causal organism may overwinter, and in particular, the precise mode of association between pathogen and seed.

In view of the considerable economic importance of Alternaria leafspot of zinnias in New Zealand, the fact that virtually all seed used for New Zealand sowings is imported, and the absence of any investigational work on the disease and its causal organism under New Zealand conditions, a study was carried out between January 1963 and November 1964 at Massey University of Manawatu, the main objectives of which were:

1. To define symptoms of the disease on seedlings and adult plants.
2. To study the morphological and physiological properties of the causal organism on culture media.
3. To determine the method(s) whereby the pathogen may overwinter.
4. To study the seedborne nature of the disease.
5. To develop a practical method whereby seed may be screened for the presence of viable inoculum.

6. To determine the health status of zinnia seed imported to New Zealand during 1963 and 1964.

7. To investigate the possibility of controlling Alternaria leafspot disease in New Zealand by use of an appropriate seed treatment.

MATERIALS AND METHODS.

The materials and methods used in each experiment are described in detail in the appropriate sections. Techniques and materials used in several sections are described here and media components outlined in Appendix I.

1. LABORATORY TECHNIQUES.

MEDIA PREPARATION.

1. Artificial media.

Stocks of potato dextrose agar (PDA), prune agar, and water agar were prepared in two litre quantities, autoclaved, and stored in 250ml. flasks (approximately 150ml per flask) stoppered with cotton wool. These media were prepared from Oxoid and Difco preparations dissolved in distilled water. Other artificial media were prepared when required for specific studies.

2. Water-soluble plant extract media.

Autoclaved and non-autoclaved.

Both were prepared from natural plant tissue, and the water extract added to 2.4% water agar. The flasks were stoppered with cotton wool and stored at 10° C until required.

3. Natural media.

Plant tissue was fumigated with chloropicrin and transferred aseptically to sterile petri plates. 1.2% water agar was poured into each plate over the plant tissue. Plates were stored at room temperature for at least ten days to allow the chloropicrin vapours to dissipate and growth substances from the plant material to diffuse through the agar.

Propylene oxide would have been a preferable sterilant to chloropicrin, but was unfortunately not available in New Zealand. Propylene oxide is a less severe sterilant, having less effect on organic plant components than chloropicrin or heat and has the advantage that plates may be poured prior to sterilisation, thus reducing the risk of contamination.

POURING OF PLATES.

The fungus was cultured in the laboratory in petri plates 9.5cm in diameter. 15 to 20ml of medium was used in each plate, allowing approximately nine plates to be obtained from the 150ml of media stored in each flask. The pouring process was carried out under normal conditions of asepsis. This involved flaming the neck of the flask and lifting the lid of the plate directly over the bottom during pouring, to reduce the chance of aerial spores contaminating the media.

METHOD OF INOCULATION OF MEDIA.

Initial difficulty was experienced in finding a suitable inoculation material for culture studies. Single spores and mycelial fragments proved unreliable and it was decided to use a circular piece of inoculum from the fringe of a scraped, 10 day culture. The piece was cut from the culture with a 5mm diameter corkborer. The disc was transferred to the plate to be inoculated using a flamed needle, and placed upside down in the centre of the plate so that the fungal mycelium was in direct contact with the agar surface.

MEASUREMENT OF COLONY GROWTH ON CULTURE.

Inoculation of plates by discs of agar allowed linear increase in growth with respect to time. Because of this linear rate it was necessary to measure the colony diameter only after a specific period - usually 10 days. Colony size was recorded in mm. Two measurements were taken, at right angles to each other, and the average recorded. In all measurements 5mm was subtracted from the total to account for the diameter of the inoculum disc.

PRODUCTION OF SPORES FOR INOCULATION AND STUDY.

Alterneria zinniae appears to be a difficult laboratory organism because of its reluctance to produce, under ordinary cultural conditions, sufficient quantities of uniform spores for inoculation experiments. The development of a suitable method for quick and certain production of spores was necessary -

1. To determine the conditions under which mass spore production could be made to occur artificially.

2. For the identification of the fungus growing from plated tissue and seed.

3. For production of adequate numbers of spores for inoculation and infection studies.

No satisfactory results were obtained from methods such as cutting up and mixing the culture in petri plates (Von Rands & Kunkel 1940); Klaus 1940 as used for Alternaria solani Ell. and Mart.). However, a modification of a method for inducing sporulation of A. solani (Ludwig, Richardson & Unwin 1962) gave fairly good results. This method consisted of scraping cultures with a glass slide and washing the culture surface under running water for twelve hours. It was found necessary to place a layer of cheesecloth over the plates to keep the medium in place during this washing process. The cultures were then cut, using a 5mm corkborer, to provide a large number of agar circles which were removed from the culture with a needle and placed mycelial face upwards on the inside of a petri plate lid. A circle of filter paper was placed on the bottom of the plate, moistened, and the lid replaced. The agar circles were held for 48 hours at room temperature to allow full production of spores along their periphery and also to a lesser extent on the top of the surface mycelium. The spores were readily removed by jetting distilled water from a plastic washbottle onto the agar circles and collecting the dislodged spores in suspension in a test tube.

This "agar circle" method, though tedious, provided spore numbers sufficient for positive identification of A. zinniae and for spore measurement studies of isolates grown under different media, temperature, and pH conditions.

In later work, however, ultraviolet light was used to provide high concentrations of spores for plant inoculation and inoculum potential studies. The culture surface was scraped with the end of a glass slide and washed in running water for 12 hours. The open plates were then exposed to ultraviolet light for one minute. They were then stacked on a tray in an inverted, slanted

position, so that each plate was partially closed by the bottom of the plate against which it leant. This arrangement appeared to provide optimum humidity conditions for sporulation. The plates were incubated at 28°C in the laboratory. A velvety layer of spores could be seen covering the agar surface within 36 hours. These were washed off using a washbottle jet. A fine camelhair brush was also used to ensure dislodgement of all spores present. The plates were thenrestacked as before. This method had the advantage over the "agar circle" method in that several successive crops of spores could be harvested at one to two day intervals until the medium became too dry. Although aseptic conditions were not maintained in this process contamination was not found to interfere with inoculation or spore germination studies.

When not required for immediate use the spores in suspension were collected on filter paper by suction through a Buchner funnel, air dried and stored in closed petri plates at 10°C. Under these conditions the spores were found to retain their viability and pathogenicity for at least six months.

The use of a detergent to help in dislodging spores was satisfactory, provided that spore concentration measurements only were required. It was not used if the resultant spore suspension was required for plant inoculation as detergents appeared to reduce spore germination and viability, and to have a delaying effect on disease manifestation in inoculated plants.

In all spore studies concentrations were expressed as spores/ml. The original suspensions were centrifuged at 1500 rpm for 10 minutes and the spores resuspended in 10ml of water to obtain comparable measurements in all cases. The concentrations obtained by the UV method were up to 350,000 spores/ml from each plate.

METHODS OF ISOLATION OF THE FUNGUS FROM HOST TISSUES.

A. Isolation from spores.

Diseased plant material was arranged on a glass slide and placed in the bottom of a petri plate which had been lined with filter paper. The filter

paper was moistened with distilled water to allow maximum humidity to develop in the petri plate when the lid was replaced. The plate containing the tissue was held at 28°C for 48 hours, and the tissue examined under a 50X binocular microscop Sporulation was invariably evident, but if the spores were not completely developed the petri plate was replaced in the incubator for a further 12 hours. In most cases an incubation period of 48 hours was sufficient to allow the full development of spores. Isolates of the fungus were then obtained by removing a single spore from the tissue surface, using a flamed needle, and placing it in the centre of a plate or test tube slope of PDA. Spores growing in regions relatively free from other contaminants were chosen for isolation. In some instances (e.g. diseased stem or root tissue) it was necessary to cut the lesioned area medianally before incubation. This induced the fungus to sporulate along the cut surface, whereas it was often loathe to sporulate on unwounded areas of the tissue. The presence of spores on the edge of a cut surface was also convenient in allowing them to be more readily removed from host tissue and lessened the risk of dislodging contaminants along with the spore.

B. ISOLATION AS MYCELIUM.

An alternative to the more usual method of isolation from tissue by single spores was to plate diseased tissue pieces to agar and allow the fungal mycelium to grow out into the medium. In most cases 0.1% mercuric chloride solution was used as a surface sterilant to kill contaminants present on the tiss before plating. An immersion period of 1 min. was found to be sufficient for thi purpose. In some cases severe washing of tissue pieces in running tap water for 6 hours was used to remove surface-borne contaminants.

Small pieces of tissue about $1mm^2$, cut from the junction of healthy and diseased tissue on the periphery of the lesion, were used for plating to agar Tissue from this area was used in an endeavour to obtain isolation pieces in which the fungus was actively growing and invading healthy plant cells. It was less likely that saprophytic fungi and bacteria had been able to colonise such areas to any great extent. The tissue pieces were placed on a muslin square and the corners of the muslin drawn together and secured with a rubber band.

The muslin bag so formed was then placed in a beaker for washing or immersed in 0.1% mercuric chloride for 1minute. The muslin bag was removed from the mercuric chloride or washing water, opened and laid out on sterile blotting paper to dry for 2 hours. During this drying period another sheet of sterile blotting paper was placed over the muslin to reduce the chance of contamination of the tissue pieces by aerial spores. When dry, the tissue pieces were transferred aseptically to prune agar. Five tissue pieces were placed on each plate. Prune agar was used for plating because its pH (pH5.5-5.7) is such as to effectively inhibit the growth of bacteria associated with plant tissue. The plates were incubated at 28°C for 3 days and reisolation of the fungus growing out of the tissue to fresh plates of PDA was then employed to obtain the fungus in pure culture.

This tissue plating technique was used in cases where the fungus was loathe to sporulate directly on host material subjected to high humidity. In all other cases, where sporulation readily occurred directly from host tissue, isolation by single spore inoculation of plates was used, since this method was less tedious and gave quicker results. The tissue plating method was especially useful for the isolation of the pathogen from infected root tissues and from debris of infected plants which had been buried in the soil for several months.

Unless specifically stated, the fungus was isolated from host tissue by means of single spores.

2. ZINNIA PRODUCTION IN THE GLASSHOUSE.

The soil used for all seed sowings in the glasshouse was steam sterilised and contained loam, peat and washed sand in the ratio 2:1:1, plus $1\frac{1}{2}$ oz. superphosphate and $\frac{3}{4}$ oz. ground limestone per bushel. Seedboxes 12" x 9" x 3" were filled with this soil to within 1" of the top and the boxes placed in open flat trays containing water to moisten the soil prior to sowing. Soil was considered to be suitably wet when patting the surface with the hand produced a slurry. The boxes were then removed from the trays and allowed to drain for ten minutes. The soil was made reasonably firm in each box and

levelled with a short piece of timber which was dragged over the soil surface. Seed was lightly scattered on the surface and covered to a depth of about $\frac{1}{4}$" with finely sifted loam. Further consolidation of the surface was then carried out to allow maximum contact between soil and seed. The boxes were lightly watered with a mist spray and placed on the glasshouse bench out of direct sunlight. Approximately 200 to 250 seeds were sown per box.

The seed germinated in about three days depending on temperature, and no further watering was carried out until full emergence had occurred (7 to 10 days). In the early stages of growth watering was carried out by misting, but when the plants were fully established the water was applied directly onto the soil surface.

Plants were pricked out when $1\frac{1}{2}$ to 2" high into a transplanting soil mixture containing loam, peat and sand (7: 3: 2), 1 oz lime/bushel and 4oz/bushel of a basic fertiliser mixture containing 2 parts superphosphate, 2 parts dried blood and 1 part sulphate of potash. In most cases transplanting was directly into pots. This operation was carried out allowing as much soil as possible to adhere to the roots.

When they were beginning to bud the plants were pinched back to allow the development of more leaves and flowers per plant.

Overwatering was avoided at all stages of growth to allow maximum root development. In general no supplementary nutrients were added to pots during the growth period.

Sowings were made every three weeks to provide sufficient numbers of plants in all stages of growth for experimentation.

3. GLASSHOUSE MANAGEMENT AND DISEASE CONTROL.

During the summer months some difficulty was experienced in keeping the glasshouse temperature down to moderate levels in the heat of the day. Misting the inside of the roof and walls, and the opening of all vents was effective to a certain extent. On very hot days (over 85° F glasshouse temperature) it was

found necessary to pour water on the floor and bench surfaces to maintain temperatures cool enough for plant growth.

In the winter, thermostatically controlled electric heaters mounted below the benches enabled the temperature to be kept above 55° F and allowed plant growth throughout the winter months.

The main insect pests present on the plants in sufficient numbers to require control measures were white butterfly larvae, aphids and red spider mite. These were controlled by Derris dust, Lindane dusts and Azobenzene "Fumite" smokes respectively. Aphids were by far the most troublesome and during the summer and autumn dusting was carried out every week. Once a fortnight a commercial size "Nexa" + strip was burnt in the glasshouse as a fumigant for general purpose insect control.

The only fungal disease of any consequence was Botrytis cinerea Pers. ex Fr. This was controlled by burning a "Fumite" bomb in the glasshouse when outbreaks occurred. No special measures for the control of A. zinniae were employed except care to prevent direct watering of leaves and the removal of any infected plant tissue. Generally the removal of the cotyledons of all plants during transplanting was sufficient to keep the disease at a low level.

After use all boxes and pots were soaked in cold water for 24 hours and then scrubbed to remove adhering soil. They were then soaked in a 1% formalin solution for 24 hours prior to stacking and subsequent reuse.

+ "Nexa": The trade name of an I.C.I. product containing lindane as its basic ingredient.

4. PLANT INOCULATION TECHNIQUES.

The following inoculation methods were successfully used in glasshouse experiments carried out over a wide temperature range (60-85° F):-

1. A drop of spore suspension was placed on the flat portion of leaves using a fine nozzled dropper. This method also proved successful in stem and flower inoculation experiments. All controls were inoculated with drops of sterile distilled water.

2. Plant parts were atomised to run-off point with a spore suspension by use of a "Windex" sprayer of the plunger type. Controls were sprayed with sterile distilled water.

3. Mycelium on blocks of agar approximately $\frac{1}{4}$" square were placed, mycelial side downwards, on the surface of leaves and stems. Controls were inoculated with clean agar squares.

All plants were then placed in a glass-walled inoculation cabinet for 48 hours following inoculation. There was no critical control of temperature and humidity during glasshouse inoculation experiments. In general the temperature was kept down by maintaining air movement through the glasshouse by leaving all vents open during the day. Humidity was kept as near to 100% as possible by spraying the plants and the interior of the inoculation cabinet with a fine spray of tap water at frequent intervals during the day. The closing down of the house and the use of electric heaters at night maintained the temperature at or above 55° F. These temperature and humidity conditions were continued for 48 hours following inoculation, and the plants were then removed from the cabinet and placed on the glasshouse bench. Recording of symptoms, percentage infection, and subsequent reisolation were carried out 10 days after inoculation, unless otherwise stated.

The three different methods of inoculation gave comparable results in preliminary experimentation. The results of this work are recorded in Table 1.

TABLE 1. COMPARISON OF METHODS OF ARTIFICIAL INOCULATION OF PLANTS.

Source of inoculum	Method of inoculation	Inoc. conc. 1000's / ml.	No. plants inoculated.	No. plants control	Total no. leaves inoc.	Total no. leaves infected	Perc. infection 10 days.	Diff. from avge. % infection.	Reisolation success.
Spores from PDA culture	1	64	6	2	48	41	87.6	-0.7	+
Spores from washed leaves previously subjected to high humidity 48hrs. 28°C.	1	60	6	2	38	32	84.2	-4.1	+
Spores from PDA culture	2	64	6	2	27	24	88.9	+0 .6	+
Spores from PDA culture	2	64	6	2	39	33	84.6	-3.7	+
Mycelium on squares of agar from 10 day culture from PDA.	3	-	6	2	41	37	90.2	+1.7	+
Mycelium on squares of agar from 10 day culture from PDA.	3	-	8	2	18	17	94.4	+6.1	+

This experiment demonstrated that there was no appreciable difference in the percentage infection obtained by each of the three methods. In most experiments methods 1 and 2 were used as they were both more convenient and more comparable to infection under natural conditions.

In plant inoculation experiments requiring critical control of both humidity and temperature, a sealed room which could be maintained at all temperatures from 65 to 85° F (± 2°) by means of a fan heater attached to a thermostat was used. Humidity was kept at levels ranging from 75% to 90% by placing shallow trays of water in front of the heater, and by placing the pots containing inoculated plants in trays of water. A thermograph was used to record the temperature and relative humidity conditions throughout critical experiments.

5. METHOD AND ASSESSMENT OF ZINNIA SEED GERMINATION.

The ultimate aim of testing for germination was to gain information on the field planting value of the seed and to provide results which could be used to compare the value of different seedlines and treatments.

Germination is defined as "the emergence and development from the seed embryo of those essential structures which are indicative of their ability to produce normal plants under favourable conditions." (Lithgow. 1956). It is thus not correct to regard the number of sprouted seeds as representing the germination of each sample, but only seeds producing normally developed healthy seedlings. In this study normal seedlings were considered to have either of the following features:-

1. Seedlings free of decay, with normally developed and attached cotyledons and roots.
2. Seedlings free of decay, presenting a normally developed root but having small portions of one or both cotyledons broken off.

Abnormal seedlings, which were not counted in the germination percentage for each seedline, were considered to have one or more of the following deformities:-

1. Poorly developed at the end of the test as a result of slow germination due to low vigour.

2. Broken sprouts, including seedlings with one or both cotyledons missing, and seedlings having a portion of the root missing and where subsequent growth of seminal roots did not occur by the time of the final count.

3. Unhealed cracks or breaks on the hypocotyl or roots, that extended into th conducting tissue.

4. Roots or leaves which were injured to such an extent that the injury prevented their normal functioning.

5. Seedlings which, at the end of the germination period, showed practically no sign of growth even though the seedcoat may have burst, regardless of whether or not the cotyledons were coloured green.

6. Weak unhealthy shoots or roots.

7. Deformities such as rolling up of the cotyledons or hypocotyl.

8. Seedlings developed abnormally owing to seedborne disease, provided that there was no evidence that infection took place from a neighbouring infected seed or seedling.

Laboratory germination of zinnia seed was carried out by the "folded towel" technique as described by the International Seed Testing Association. The germination temperature was 25°C, and interim and final counts were made 3 days and 7 days respectively after the commencement of the test.

Two blotters, each with 100 seeds, were used for each sample tested. Folded blotting paper was used as the substrate, the seed being spread sufficiently to prevent the seedlings from coming into contact before they were counted and removed. The folded blotters were enclosed in moistened towelling to maintain the level of moisture needed to supply the requirements of the seed. The towelling and enclosed blotters were placed on a sheet of plastic which served as a base to support the sample during the test period.

Most of the germination tests were carried out at the Seed Testing Station, Palmerston North, but some tests were carried out at Massey University of Manawatu, by using a 25°C incubator with a glass door as a germination cabinet.

In all cases the average number of normal seedlings obtained from each of two lots of 100 seeds was recorded as the "germination percentage".

C H A P T E R I.

T H E F U N G U S.

Introduction.

The morphological and physiological characteristics of A. zinniae isolated from naturally infected zinnia plants in New Zealand were studied on artificial media in the laboratory. Special consideration was given to conidial production by the fungus on different culture media to determine whether any of the media studied allowed reliable and prolific production of conidia which could be used for plant inoculation and disease cycle studies.

Inoculation studies were undertaken to determine whether A zinniae was pathogenic to all parts of plants of Zinnia elegans (roots, stems, cotyledons, adult leaves, blossoms and the seedcoat). This study was felt necessary because there does not seem to be any work to date proving the pathogenicity of A. zinniae to all these host organs. Proof of pathogenicity was determined using Koch's Postulates and supplemented by spore measurement studies.

The taxonomy and nomenclature of the fungus causing Alternariosis of zinnia were also considered.

A. ARTIFICIAL MEDIA STUDIES.

1. INFLUENCE OF MEDIA TYPE ON CULTURAL FEATURES AND CONIDIAL PRODUCTION OF A. ZINNIAE PAPE.

Species of the genus Alternaria usually grow well on artificial media. A. zinniae is no exception, but there are reported instances where difficulty has been experienced in inducing sporulation of the fungus on culture media (Dimock & Osborn 1943, McDonald & Martens 1963). Dimock & Osborn report that "Sporulation has been very poor on all culture media tested, including potato-extract agar, PDA, bean agar, oatmeal agar, pea agar, cornmeal agar and water agar." Neergaard (1945) also notes the difficulty of maintaining sporulation of isolates of A. zinniae after several weeks growth on artificial media.

Accordingly, studies were undertaken to determine which media were most suitable for (a) growth, and (b) sporulation of A. zinniae.

A total of 18 autoclaved media, 6 natural media and 7 plant extract media were investigated and the morphology and sporulation capacity of the fungus on

each media type recorded.

To avoid altering the composition of natural media by subjecting them to high temperatures by autoclaving, chloropicrin was used as a surface sterilant. Propylene oxide has been used overseas (Klarman & Craig 1960) for this purpose, but was unfortunately not available in New Zealand.

Snyder & Hansen (1947a, 1947b) outlined the various methods of using natural host material as media for fungal culture.

Details of the preparation of the 31 different media used in these studies are recorded in Appendix 1.

(a) Artificial media.

All artificial media were prepared in 250 ml lots sometime before they were needed and stored in flasks in the refrigerator. When required they were heated in a steam bath until liquefied. Before pouring into petri plates the media was cooled in a water bath at 45-50° C.

(b) Natural media.

The advantages obtained from the use of natural media may be due to small amounts of essential vitamins and growth substances present in the natural plant material used.

Natural media were prepared from plant material, which was suspended in 1.2% water agar. Pieces of plant tissue were placed in a dessicator containing 2 ml. of chloropicrin for 24 hours. The material was then transferred to sterile petri plates and covered with 1.2% water agar. The agar was allowed to solidify and the plates stacked in an inoculation cabinet for 10 days at room temperature. This allowed dissipation of any chloropicrin vapour from the plates and also allowed time for the diffusion of any organic material or growth substances from the surface of the plant tissue into the agar.

(c) Plant extract media.

Natural plant material was placed in a Waring blender and finely macerated in 50 ml. of distilled water for 15 minutes at 2,500 rpm. The resultant slurry was filtered through two layers of cheesecloth to remove

most of the fibrous material and then put through blotting-paper-mache filter in a Buchner funnel. The total volume was then increased to 200 ml. using sterile distilled water. The suspension was filtered aseptically through a Seitz-Werner filter to remove any fibrous material and also any bacterial and fungal contaminants. The filtrate was transferred aseptically to an autoclaved flask containing 100 ml. 1.2% water agar, and the flasks stoppered with cotton wool. This allowed the preparation of water soluble components from natural plant tissue in water agar. When required the flasks were heated in a steam-bath to liquefy the agar and the medium held at 45-50° C. in a waterbath ready for use.

Petri plates were labelled with a random number corresponding to each medium type. Approximately 20 ml. of medium was poured into each of 5 plates and allowed to solidfy at room temperature. Each plate was inoculated with a 5mm circle of the fungus obtained from the periphery of a 10 day old culture of A. zinniae grown on PDA.

Following inoculation the plates were incubated at 28° C. Average measurements of colony diameter were recorded for each media after 10 days. Results of cultural characteristics and conidial production were also recorded. Experiments were repeated until uniform results were obtained.

Average colony diameter and description of colony characteristics are presented in Table 2.

TABLE 2. MORPHOLOGICAL CHARACTERISTICS OF 10 DAY OLD CULTURES OF *A. ZINNIAE* ON DIFFERENT MEDIUM TYPES.

TYPE OF MEDIUM.	COLONY DIAMETER	COLONY COLOURATION Lower surface	Uppersurface	MYCELIUM DENSITY	COLONY SHAPE	COLONY OUTLINE	AERIAL MYCELIUM	pH
			Centre fringe					
A. ARTIFICIAL AUTOCLAVED MEDIA.								
Difco P.D.A.	78	B	DG.	W	4	R	E	4	5.
Oxoid P.D.A.	84	B-Br.	DG	W	3	R	E	4	5.
Lab. P.D.A.	86	B	DG.	W	4	R	E	4	5.
Prune agar	79	Br.	Br.	W	3	R	D	2	5.5
Water agar	80	W	W	W	1	R	D	0	4.8
Nutrient agar	71	W	W	W	3	R	D	5	6.7
Czapek-Dox agar.	82	B	G-GR	W	4	R	E	3	6.6
Mycological peptone agar.	45	Br.	G-GR	W	4	R	D	2	5.6
Sabouraud agar.	43	B	G-GR	W	5	R	E	2	5.5
Cornmeal agar	74	B	DG	W	4	R	E	1	5.4
Soil agar.	82	W	W	W	2	R	D	0	6.2
Malt agar	48	B	G	W	4	R	E	4	5.9
Oatmeal agar	86	Br.	Br.	W	3	R	E	2	6.3
Tomato-juice agar	64	B	G-GR	W	3	R	E	4	5.6
T.G.Y.E. agar	62	B	G-GR	W	3	I	D	3	6.2
V8-juice agar	71	B	LG	LG	5	R	E	5	4.3
Dextrose agar	84	LBr.	LG	W	2	R	E	2	6.8
G.L.P.A.	34	B	G	W	5	R	E	5	6.1

(Continued over)

TABLE 2 (contd.)

Type of MEDIUM.	COLONY DIAMETER	COLONY COLOURATION Lower Surface		Uppersurface	MYCELIUM DENSITY	COLONY SHAPE	COLONY OUTLINE	AERIAL MYCELIUM	pH.
		Centre	Fringe						
B. NATURAL MEDIA.									
Zinnia blossom agar	84	B	G-GR	W	1	I.	D	1	
Zinnia leaf agar	87	W	W	W	1	I	D	1	
Zinnia seed agar	46	B	DG	W	2	I	E	3	
Tobacco leaf agar	71	B	G	W	2	R	E	3	
Tomato leaf agar	76	B	LG	W	3	I	E	4	
Lettuce leaf agar	77	B	G	W	3	R	E	2	
C. PLANT EXTRACT MEDIA.									
Zinnia blossom agar	83	B	G-GR	W	2	R	D	2	6.6
Zinnia leaf agar	84	B	G-GR	W	1	R	D	2	6.9
Zinnia seed agar	68	W	W	W	1	R	D	2	6.6
Tobacco leaf agar	65	B	G	W	3	R	E	4	6.4
Marigold leaf agar	84	B	DG	LG	2	R	E	2	6.2
Tomato leaf agar	47	W	W	W	2	R	E	3	5.3
Lettuce leaf agar	88	G	G	W	2	R	E	2	6.4

KEY (Table 2)

COLONY COLOURATION: B = black, Br. = brown, G = grey
GR = greenish tinge, D = dark, L = light

MYCELIAL DENSITY: Represented on a scale 1 to 5, where the mycelium density on Difco P.D.A. = 4.

COLONY SHAPE: R = regular, I = irregular.

COLONY OUTLINE: E = entire, D = diverse.

AERIAL MYCELIUM PRODUCTION: Represented on a scale 1 to 5, where the aerial mycelium production on Difco P.D.A. = 4.

The colour of colonies on most media was grey. The fringes of all colonies were lighter coloured than the centres of the cultures, and were often white. Some media supported abundant fluffy white aerial mycelium whereas others tended to favour mycelial growth below the surface of the substrate.

Most colonies were circular in shape but some showed a diverse outline. Some of the colonies growing on natural media showed irregularity in shape, possibly due to an uneven distribution of organic plant materials in the media favouring growth.

Some bacterial contamination was present in natural media, which tended to decrease colony size on some of the media prepared from plant tissue treated with chloropicrin. As a result, colony diameter measurements recorded on these media are probably less than that which would occur if full growth-rate potential could be expressed in the absence of contaminants.

Results of the relative conidial production of colonies of A. zinniae on each medium type were recorded by counting the average number of spores in each of three random fields under a binocular microscope (X 40) for each of three different areas on each colony - centre, mid-region and fringe. The total spore production on each medium was determined by washing the culture surface, after 10 days growth at 28° C, with a fine jet of water and determining the concentration of spores in a total volume of 10 mls. of water with a haemocytometer.

Results of conidial production on each medium type are recorded in Table 3.

TABLE 3. CONIDIAL PRODUCTION OF A. ZINNIAE ON DIFFERENT MEDIUM TYPES.

TYPE OF MEDIUM	LOCATION of CONIDIA	AERIAL OR SURFACE MYCELIUM	AVERAGE NO. OF CONIDIA C	M	F	TOTAL CONIDIAL PRODUCTION 1000/n
A. ARTIFICIAL AUTOCLAVED MEDIA.						
Difco P.D.A.	Ov	A	63	38	46	9
Oxoid PDA	Ov	A	15	12	20	6
Lab. P.D.A.	C	A	62	0	0	6
Prune agar	C	S	16	3	0	4
Water agar	C	S	10	0	0	4
Nutrient agar	Nil	Nil	0	0	0	0
Czapek-Dox agar	C	A	48	7	5	4
Mycological peptone agar	Ov	A	52	61	76	6
Sabouraud agar	M	A	32	200+	20	22
Cornmeal agar	Ov	A	6	11	2	2
Soil agar	C	S	15	2	0	2
Malt agar	F	A	0	0	14	2
Oatmeal agar	Ov	S	12	9	4	4
Tomato juice agar	C	A	60	19	0	8
T,G.Y.E. agar	C.M.	A	200+	42	0	20
V8-juice agar	C	A	16	17	0	6
Dextrose agar	Nil	Nil	0	0	0	0
G.L.P. agar	Nil	Nil	0	0	0	0

(Continued over.)

TABLE 3 (Contd.)

TYPE OF MEDIUM	LOCATION OF CONIDIA	AERIAL OR SURFACE MYCELIUM	AVERAGE NO. OF CONIDIA C	M	F	TOTAL CONIDIAL PRODUCTION 1000/ml.
B. NATURAL MEDIA.						
Zinnia blossom agar	Ov	S	161	113	10	36
Zinnia leaf agar	Ov	S	200+	161	17	48
Zinnia seed agar	Ov	S+A	112	183	42	28
Tobacco leaf agar	Ov	S+A	200+	110	200+	48
Tomato leaf agar	Ov	S	200+	200+	23	40
Lettuce leaf agar	Ov	S+A	130	200+	200+	60
C. PLANT EXTRACT MEDIA.						
Zinnia blossom agar	Ov	S	18	200+	30	18
Zinnia leaf agar	Ov	S	37	200+	57	16
Zinnia seed agar	Ov	S	118	16	45	12
Tobacco leaf agar	Nil	Nil	0	0	0	0
Marigold leaf agar	C	A	37	3	0	4
Tobacco leaf agar	C	A	16	0	0	2
Lettuce leaf agar	C	S	23	2	0	2

KEY : LOCATION OF CONIDIA. Ov = overall
C = centre
M = mid-region
F = fringe

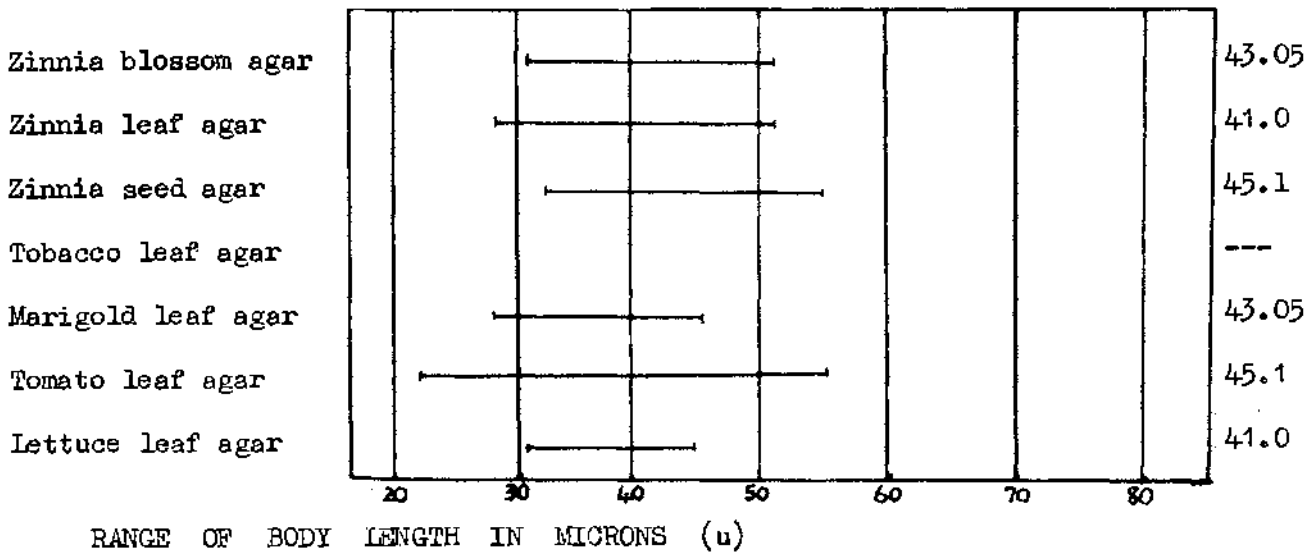
Total conidial production was dependent on the total colony area, mycelial density, and the relative density of conidia over the culture surface.

To determine whether medium type had an effect on the dimensions of conidia produced by the fungus, thirty spores from each medium were measured on a stage micrometer. The range of spore body length, body width, beak length and total length are recorded in Appendix II. The body length of spores of A. zinniae produced in each medium type is recorded graphically in Figure I.

FIGURE 1. BODY LENGTH OF CONIDIA OF A. ZINNIAE FROM DIFFERENT MEDIA.

FIGURE 1 (contd.)

C. PLANT EXTRACT MEDIA.



From these results it can be seen that, of the 31 different media studied, only a few favoured high growth rate of the fungus, i.e. -

Autoclaved media	Oxoid PDA
	Lab. PDA
	Czapek-Dox agar
	Oatmeal agar
	Soil agar
	Dextrose agar
Natural Media	Zinnia blossom agar
	Zinnia leaf agar
Plant extract media	Zinnia blossom agar
	Zinnia leaf agar
	Marigold leaf agar
	Lettuce leaf agar

Colonies on all of the media studied were non-chromogenic, except on Lab. PDA where one of the five non-pigmented inoculation pieces gave rise to a chromogenic culture.

Moderate numbers of spores of the fungus were produced on colonies grown on: Autoclaved media:

Sabouraud agar, TGYE agar.

All natural media

Plant extract media: Zinnia blossom agar, zinnia leaf agar.

Natural media supported greatest spore numbers of the fungus from each colony, but their preparation was not considered practical for laboratory studies of *A. zinniae* or for the production of conidia of the fungus for plant inoculation experiments.

In later studies on methods of inducing sporulation in culture (page 32) ultraviolet light treatment of colonies growing on Lab. PDA was found to be of most value. This medium also has the advantage of being readily available, inexpensive and simple to prepare.

Spore measurements varied considerably with the media on which they were produced. Spore body length was most consistent between medium types on plant extract media, but varied quite considerably on different autoclaved and natural media.

The overall range of body length of conidia of the fungus on all media was 18.45 u to 82.0 u with a mean of 47.18 u.

CONCLUSIONS.

The results of this experiment show that A. zinniae is capable of growth on a wide range of artificial, natural and plant extract media. Oatmeal, dextrose and potato-dextrose agars appear to be the most convenient for laboratory study of the fungus in culture.

For spore production on culture media, Sabouraud and TGYE agars appear to be the most practical. On only 4 of the 31 media studied did the fungus fail to produce at least some conidia in culture. In most cases, however, (23 of the medi. studies), conidial production was so low that these media could not be used for the production of spores for plant inoculation or disease cycle studied.

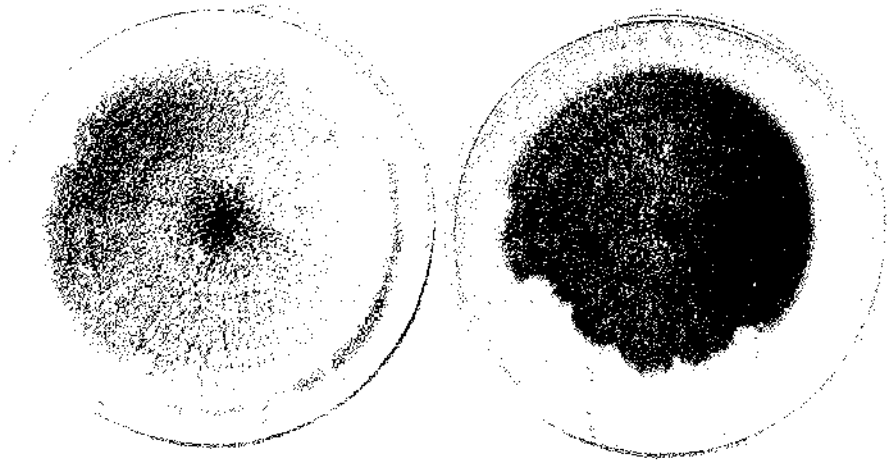


PLATE 1. Two cultures of A. Zinniae on PDA.

Left - reverse side of culture after 8 days growth at 28°C.

Right - obverse side of culture after 10 days growth at 28°C.

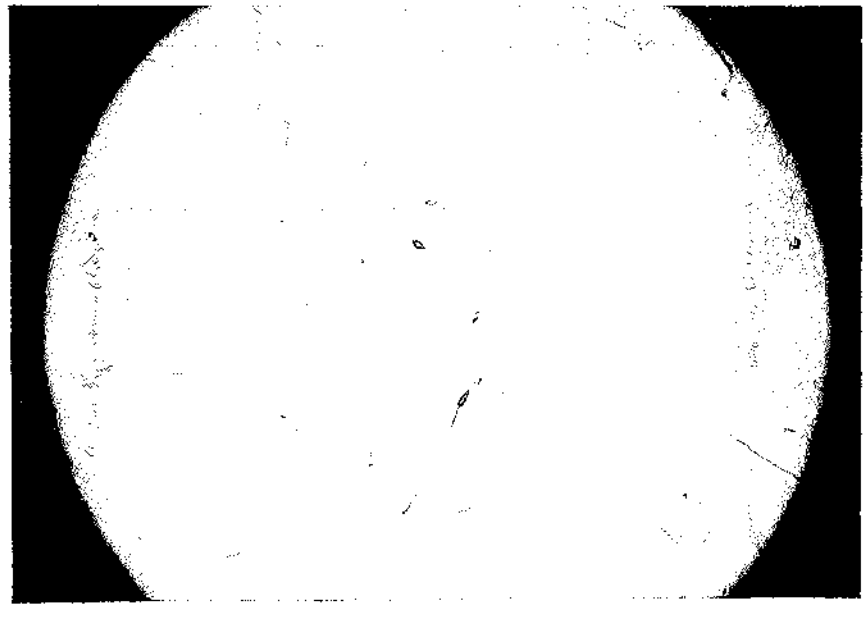


PLATE 2. Microphotograph of surface of culture of A. zinniae on PDA showing sparsity of conidial production.

2. EFFECTS OF TEMPERATURE ON THE GROWTH OF A. ZINNIAE IN CULTURE.

Studies were undertaken to determine the effect of temperature on colony growth characteristics and conidial production of the fungus. This study was also used to determine the range of temperature for optimum growth of the fungus on culture media.

Two isolates of A. zinniae, one from field infected leaves and the other from naturally infected blossoms, were used to inoculate plates of PDA. Thirty-nine plates per isolate were inoculated with a 5mm agar circle from the periphery of each culture, and the plates incubated at different temperatures. Three plates of each isolate were placed in incubators set at temperatures of 14, 20, 22, 26, 28, 30, 33, 37, 40, and 45° C, and three plates in refrigeration units at 0 and 5° C. The diameter of the colonies was measured after 10 days and the average for each temperature calculated for each isolate.

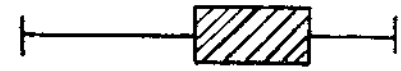
The differences in colony diameter after incubation for 10 days were not significantly different between the two isolates used. The average colony diameters for each isolate are recorded graphically in Figure 2.

The cardinal temperatures for growth of the fungus on PDA were 0, 28 and 45°C. The fungus grew well over a range of temperature from 25 to 30 ° C.

As far as colony characteristics were concerned there were marked differences in colour and aerial mycelium production, depending on the temperature at which the fungus was grown. At optimum temperatures for growth, colonies were grey to grey-green in colour with a white fringe area. Higher and lower temperatures induced changes in cultural features in that colonies growing on plates incubated at low temperatures possessed a wider fringe area, dense, fluffy, aerial mycelium, and tended to be lighter in colour (white to light grey). Cultures arising from inoculated plates incubated at temperatures above the optimum possessed a very narrow fringe area, produced spafse aerial mycelium and tended to be darker in colour (dark grey to black). Thus as the temperature of incubation of inoculated

FIGURE 2

EFFECT OF TEMPERATURE ON GROWTH RATE AND CONIDIAL PRODUCTION



RANGE OF CONIDIAL PRODUCTION

ISOLATE FROM LEAF

ISOLATE FROM BLOSSOM

COLONY DIAMETER

100
90
80
70
60
50
40
30
20
10
0

5 14 20 22 24 26 28 30 33 37 40 45

TEMPERATURE °C

plates increased from 0° C to 45° C, aerial mycelium production and fringe diameter were decreased and the intensity of mycelium pigmentation increased from light grey to black.

Conidia were produced in low numbers on the surface of colonies grown at temperatures ranging from 20°C to 33°C, with most spores being present on cultures at temperatures between 26 and 30° C. Conidial production at all temperatures was relatively poor, maximum concentrations of only 6,000 spores per ml being obtained at 28°C and less than 2,000 spores per ml at 20 and 33°C.

The optimum temperature for growth of A. zinniae in culture has been recorded by various workers. Neergaard (1945) suggests an optimum of 27°C on standard nutrient agar. Dimock & Osborn (1943) state 27°C on PDA, and Kispatic (1951) suggests that the optimum temperature for growth and sporulation of the fungus on malt agar and malt-peptone agar is "about 25°C". The results by these authors are in reasonable agreement with those obtained in the present study.

The temperature required by the isolates for mycelial growth may be made the subject of taxonomic considerations, as it may imply a differentiation of physiological races within the individual species or variety (Neergaard 1945). No such races were distinguishable on the basis of different temperature optimums in culture between the isolates of A. zinniae studied here.

CONCLUSION.

On the basis of this study it was concluded that the optimum temperature for growth of A. zinniae in the laboratory was 28°C. This temperature was accordingly used for incubation of the fungus on culture media.

The temperature range allowing sporulation was narrower then the range for growth. A small temperature increase above the optimum for growth had a greater depressing effect on spore production in culture than a small reduction in temperature below the optimum.

3. THE EFFECT OF LIGHT ON COLONIES.

To determine whether light had an effect on conidial production of A. zinniae in culture, colonies of the fungus were subjected to different light intensities for varying periods of time.

Under natural conditions many fungi fruit only when exposed to light, often to the direct rays of the sun, for varying periods of time. Short periods of exposure to ultraviolet light are known to promote intense sporulation in some fungi (Leach 1962). Similarly, artificially induced alternating cycles of 12 hou light and 12 hours dark have been used to induce spore production of some fungi o culture media. (Lilly & Barnett 1951).

To study the effect of light treatment on sporulation of A. zinniae, 21 five-day-old colonies of the fungus on PDA were placed on a window-sill in the laboratory for approximately 6 hours per day, for periods from 1 to 7 days. The plates were thus exposed to sunlight which passed through the window and petri plate lid. The plates were returned to the 28° C incubator following each daily treatment. Conidial production was determined by washing spores from the culture surface with a fine jet of water and determining the concentration of the resultant spore suspension using a haemocytometer. Spore concentration was expressed as the number of spores per ml in the total volume of 10 mls. of spore suspension.

No stimulation of conidial production was observed in cultures exposed to sunlight for 6 hours each day for up to 7 days.

The effect of alternating cycles of 12 hours light and 12 hours dark on sporulation of A. zinniae in culture was also studied. Five-day-old cultures were placed under a single tube "daylight" fluorescent lamp for 12 hours and then placed in a closed drawer in the dark for 12 hours.

No increase in spore production was observed of the fungus exposed to alternating cycles of 12 hours light and dark for periods of up to 7 days.

Diener (1952) and Leach (1961) have described the use of ultraviolet light for inducing the sporulation of diverse fungi in culture.

Cultures of A. zinniae with lids removed were placed 4" from a WL30 ultraviolet light tube (peak radiation 3700 Å) for periods of exposure as low as one minute, and then the cultures were held at 28° C for 36 hours.

A very marked increase in the number of conidia produced was observed. The effect of ultraviolet light in inducing sporulation of A. zinniae and the effect of various culture treatments prior to irradiation with ultraviolet light are discussed in a later section (page 35).

4. THE EFFECT OF REFRIGERATION ON COLONIES.

In order to study the effect of low temperature treatment on the viability of *A. zinniae* in culture, two 10-day-old colonies were placed at each of three temperatures (-8, 0, and 5°C) in refrigerators. At 10 day intervals a 5mm circle of agar and mycelium was removed from cultures held at each temperature and placed in the centre of a fresh plate of PDA. These plates were placed in an incubator at 28° C, and average colony diameter recorded after 10 days.

The fungus proved very resistant to low temperatures, being capable of growth at 28°C following up to 90 days storage at -8° C. At temperatures of 0 and 5° C *A. zinniae* showed mycelial growth at 28° C following periods of storage of up to 120 days and over 312 days respectively. Tests on cultural viability following storage at 5° C were discontinued after approximately 10 months (312 days) at the end of which time the fungus was still capable of growth when incubated at 28° C.

Storage of colonies at temperatures from -8° to 0° C stopped the growth of the fungus but failed to induce any morphological changes in the cultural characteristics of subsequent colonies arising from inoculated plates incubated at 28° C. Once the colonies were returned to favourable temperatures normal growth resumed. Colonies stored at 5° C produced dense white aerial mycelium after several days, but colonies obtained by inoculating fresh plates of PDA with mycelium of cultures stored at 5° C were morphologically identical with normal cultures of the fungus.

5. FACTORS INFLUENCING SPORULATION IN CULTURE.

A study of the factors influencing the sporulation of *A. zinniae* in culture was necessary to obtain a method for the production of adequate quantities of spores for plant inoculation and disease cycle studies, and for the identification of the fungus growing out from plated tissue and seed.

Failure to sporulate in culture is a major problem in the study of any fungal pathogens. This is particularly true in the identification of seedborne fungi where the difficulty of identifying non-sporulating colonies on culture media is a major obstacle to developing routine detection procedures.

The use of different cultural media did not seem to yield useful results (Page 26). Although spores of the fungus were produced on s wide range of media, spore concentrations were insufficient for plant inoculation or disease cycle studies.

From preliminary studies it appeared that promotion of spore production, because it could not be induced by nutritional means, could only be brought about by alteration of physiological or environmental factors. The methods described by Von Rands & Kunkel (1940) and Klaus (1940) for inducing sporulation of *Alternaria solani* by cutting and mixing the culture did not give satisfactory results in inducing sporulation of *A. zinniae* in culture.

In some cases spores could be found on old, dry cultures which had been subjected to physiological shock by low temperature or heat treatment, but the numbers of spores produced were so low that they were useful only for preparation of single spore cultures, and definitely not for the production of infection material.

SURFACE WOUNDING METHOD.

Studies were carried out to determine whether physiological shock by wounding the surface mycelium of the fungus would stimulate spore production. The method employed was based on a technique described for inducing sporulation of *A. solani* on culture media by Ludwig, Richardson & Unwin (1962).

The surface of 10-day-old cultures of A. zinniae on PDA were scraped with the end of a glass slide to remove the aerial mycelium, and washed under running tap water for 12 hours. The plates were covered with cheesecloth to keep the media in place during washing. Following washing the plates were stacked on a tray in an inverted, slanted position so that each plate was partially closed by the bottom of the plate against which it leant. This arrangement appeared to provide optimum humidity for spore production.

Moderate numbers of spores were produced at the centre and periphery of cultures after 48 hours. Spore suspensions were obtained by washing spores from the culture surface with a fine jet of water to a total volume of 10 ml. and spore concentration determined using a haemocytometer. Maximum spore numbers of 20,000 spores per ml. were obtained from each culture by this method. The age of colonies treated in this way had no appreciable effect on sporulation capacity.

Although this method did not allow the production of high spore numbers for plant inoculation studies it showed that wounding of the surface mycelium was an important factor in inducing the fungus to sporulate. The washing process removed much of the pigment in the culture media of chromogenic cultures.

Thus it was concluded that the stimulation of spore production may have been due to the removal of an "unidentified anti-sporulating factor" during the washing process, as suggested by Ludwig et al. (1962) for A. solani, or to a stimulation of sporulation by the physiological shock of wounding the surface mycelium.

AGAR CIRCLE METHOD.

A further method, incorporating the technique described above and the use of a corkborer to provide small circles of agar and mycelium, was studied.

Cultures of A. zinniae on PDA were scraped as described above. The cultures were then cut with a 5 mm. corkborer to give a large number of agar circles which were removed from the culture with a needle and placed, mycelial face upwards, on the inside of a petri plate lid. A circle of moist filter paper was placed in the bottom, and the plates held at room temperature for 48 hours.

Abundant sporulation occurred around the periphery of each agar circle and to a lesser extent on the surface mycelium.

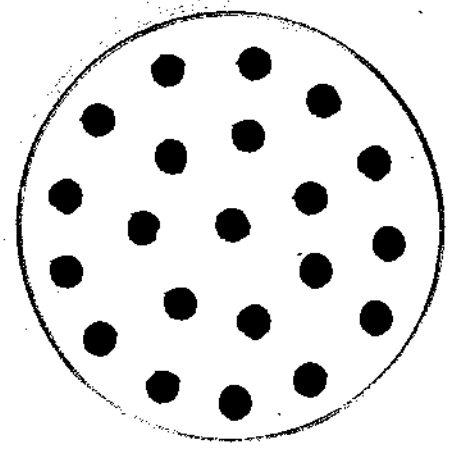
The conidia, were readily removed with a fine jet of water and the spores were collected in a test tube. The maximum spore concentration obtained by this method from a single culture was 100,000 spores/ml.

This agar circle method, although tedious, could be used to provide spore numbers sufficient for positive identification of colonies of A. zinniae arising from plated tissue and from seed plated to agar. The method also provided sufficient spore numbers for conidial measurement studies of isolates grown under different environmental conditions, and for plant inoculation studies.

It should be noted also that moderate spore numbers, up to 30,000 spores per ml, were obtained by this method on cultures which had not been scraped prior to corkboring. The scraping of the cultures removed the aerial mycelium which tended to trap conidia produced on the agar circles and thus markedly reduced the number of conidia dislodged by washing with water.

The "agar circle" method was used in all further studies to provide positive identification of A. zinniae growing out on culture media from plated tissue or seed.

PLATE 3. INDUCING SPORULATION OF A. ZINNIAE BY THE AGAR CIRCLE METHOD.



ULTRAVIOLET LIGHT METHOD.

Several workers have described the use of ultraviolet light for inducing sporulation of diverse fungi in culture (Lilly & Barnett 1951, Diener 1952, Leach 1961, 1962). Leach, (1962), states that the region of the spectrum which stimulates sporulation of many fungi has still to be defined clearly, although the blue-ultraviolet region seems to be the most active in this phenomenon. Lilly & Barnett (1951) suggest that of the factors affecting the response to ultraviolet light irradiation of spore production of many fungi in culture, length of exposure is of major importance. In addition, the age of culture could also modify the results.

A single tube WL 30 fluorescent lamp was used for these studies. The emission of the lamp showed a wavelength range of 3,000 to 4,500 Å, with maximum emission between 3,300 and 4,000 Å , and a peak of 3,700 Å.

Studies were carried out to determine whether ultraviolet light stimulated spore production of A zinniae in culture, and also to ascertain the optimum conditions of treatment for maximum sporulation. Factors considered were:-

(a) Length of irradiation period.

(b) The effect of scraping the culture surface prior to exposure to ultraviolet light.

(c) The temperature of incubation of cultures following irradiation.

(d) The effect of age of cultures irradiated on subsequent conidial production.

(e) The effect of continuous exposure and of cyclical exposure of cultures to ultraviolet light for periods of up to 7 days.

(a) Length of Irradiation Period.

Isolates used for this study were 10-day-old cultures of A. zinniae on PDA. The surface of each culture was scraped with the end of a glass slide to remove all aerial mycelium. The plates, with lids removed, were placed on a rack 4 inches from the fluorescent tube and the surface of the cultures irradiated for periods ranging from 0.25 minutes up to 10 minutes. The plates were stacked as in the surface wounding method, at room temperature. A velvety layer of spores could be seen covering the agar surface within 36 hours. These

were washed off with a fine jet of water, using a camelhair brush to help dislodge the spores. The concentration of spores in a total volume of 10 ml. of distilled water was determined using a haemocytometer. The plates were then restacked as before. Non-irradiated scraped cultures were used as controls.

The results of spore concentration measurements obtained by irradiation of 20-day-old cultures with ultraviolet light for periods from 0.25 to 10 minutes are presented in Table 4.

TABLE 4. SPORE CONCENTRATIONS OBTAINED FROM IRRADIATED CULTURES.

Exposure time - minutes.	Spore concentration in thousands per ml. (average of three colonies.)
0 (control)	18
0.25	224
0.5	272
1	386
2	262
3	247
4	212
5	182
10	178

These results indicate that irradiation of cultures by ultraviolet light greatly stimulated sporulation of the fungus and that maximum spore numbers were produced following an irradiation period of 1 minute.

(b) The effect of wounding the surface mycelium of colonies prior to irradiation on subsequent sporulation capacity of A. zinniae in culture.

Six plates of 10-day-old colonies of A. zinniae on PDA were used for each test. Three plates were scraped with the end of a glass slide prior to irradiation and the remaining three plates were not scraped. Cultures were irradiated for one minute and held at room temperature for 36 hours before spore concentratio

was determined as previously described. Scraped and unscraped cultures which were not irradiated with ultraviolet light, but held at room temperature for 36 hours, were used as controls.

The results, an average of 3 plates per treatment, are recorded in Table 5.

TABLE 5. EFFECT OF SCRAPING OF CULTURES PRIOR TO ULTRAVIOLET IRRADIATION.

Pretreatment	Length of exposure to UV light.	Spore concentration thousands per ml.
Nil	Nil	6
Nil	1 minute	36
Scraped	Nil	22
Scraped	1 minute	218

These results show the marked stimulation of sporulation by ultraviolet light treatment of scraped cultures compared to unscraped cultures.

(c) Effect of temperature of incubation of cultures following irradiation.

To determine whether the temperature of incubation following irradiation affected the number of spores which developed, 10-day-old cultures of the fungus were scraped with a glass slide, exposed to ultraviolet light for 1 minute and incubated at various temperatures for 36 hours. Three colonies on PDA were incubated at each of 8 temperatures, ranging from 10 to 50° C. Spore numbers per culture were determined as before. Non-irradiated, scraped cultures were incorporated as controls.

Results are presented in Table 6.

TABLE 6. EFFECT OF INCUBATION TEMPERATURE ON SPORE PRODUCTION.

Temperature °C.	Spore concentration (average of 3 colonies) thousands per ml. Irradiated.	Non-irradiated.
10	4	0
20	72	4
25	128	16
28	224	22
31	232	20
37	18	6
40	12	0
50	2	0

From these results it is concluded that incubation temperature following ultraviolet light irradiation is an important factor in determining spore numbers produced. In this respect incubation temperatures of 28 to 31°C appear to be the most beneficial.

(d.) Effect of culture age on sporulation of colonies exposed to ultraviolet light.

The cultures used in this experiment were grown on PDA and varied in age from 5 to 36 days. The surface of each colony was scraped with the end of a glass slide. The cultures were exposed to ultraviolet light for 1 minute and incubated at 28°C for 36 hours. Scraped, non-irradiated cultures were used as controls.

Spore concentrations per colony were determined as before and the results expressed as an average of three plates.

Results are recorded in Table 7.

TABLE 7. EFFECT OF AGE OF CULTURE ON SPORE PRODUCTION.

Age of culture. Days.	Spore concentration. Thousands per ml. 1 min. UV.	No UV.
5	18	9
7	61	16
10	130	29
14	134	32
21	92	30
36	88	31

From these results it was concluded that the maximum response to ultraviolet light treatment could be obtained by the use of cultures which were between 10 and 14 days old.

(e) Effect of continuous and cyclical exposure to ultraviolet light.

One set of colonies were exposed to ultraviolet light continuously for 7 days, while another set was exposed to an alternate cycle of 12 hours ultraviolet light and 12 hours darkness over a period of 7 days.

Scraped, 5-day-old cultures on PDA were used for each test.

Under continuous exposure colonies were fairly uniform in colour and texture, and zonation was absent. Under cyclical exposure zonation of colonies was pronounced. This zoning resulted from effects of alternating light and darkness on young, actively growing peripheral mycelium. Those zones of mycelium which had been in a peripheral position during periods of irradiation showed heavier spore production than mycelium which was at the periphery during the dark period.

The results of spore production from colonies exposed to continuous or alternating ultraviolet light for 7 days are presented in Table 8.

TABLE 8. EFFECT OF CONTINUOUS AND CYCLICAL EXPOSURE TO ULTRAVIOLET LIGHT.

Treatment	Spore concentration (average of 3 plates). Thousands per ml.
Ultraviolet light exposure 1 minute (control)	282
7 days continuous irradiation	186
7 days cyclical exposure 12 hour cycles	109

The conclusion obtained from this experiment is that neither continuous irradiation nor cyclical exposure of cultures, for up to 7 days, were as effective in stimulating sporulation of A. zinniae as a 1 minute exposure. The shorter exposure time is also far more convenient for treatment of a large number of colonies.

CONCLUSION.

As a result of the various experiments conducted to determine the optimum conditions for obtaining maximum sporulation of A. zinniae by ultraviolet light treatment, the following conditions have been shown to give maximum sporulation:-

Age of culture	10 to 14 days
Pretreatment	Surface scraping of culture
Irradiation time	1 minute
Method incubation	Inverted overlapping plates
Incubation temperature	28°C to 31°C.
Length of incubation	36 hours.

Although aseptic conditions were not maintained during the scraping, irradiation and incubation processes, contamination was not found to interfere with plant inoculation or spore germination studies.

The ultraviolet light method was quick, reliable and simple to use. It gave greater spore numbers than could be obtained by previous methods. One other major advantage of the method was that, if plates were restacked following spore collection, several successive crops of conidia could be obtained from each colony at 1 to 2 day intervals until the medium became too dry.

Because of the success of the use of ultraviolet light treatment in stimulating intense sporulation of A. zinniae on culture, the method was used throughout these studies for the production of inoculum for plant inoculation and disease cycle studies.

6. PIGMENTATION.

Some isolates of *A. zinniae* produced by single spore transfers from plant tissue subjected to high humidity produced pigments in culture media. These pigments diffused into the medium and ranged from yellow to dark red in colour. Subsequent isolations from chromogenic colonies often gave rise to non-chromogenic cultures.

Pigmented colonies consistently produced lower numbers of spores than unpigmented colonies. Freshly isolated chromogenic cultures showed higher growth rate at 28°C but did not retain their viability in cultures as long as non-chromogenic isolates.

Dimock & Osborn (1943) record that "A reddish pigment diffuses into the medium as the culture ages" and the formation of this pigment was demonstrated to be dependent on the presence of sugars in the culture medium.

Lilly & Barnett (1951) state that pigment production by a fungus is in part determined by genetic factors and in part by the environment. Nutritional factors may also modify the production of pigments by fungi in culture.

In the present study some isolates showed intense pigment production when passed through different hosts. When spores from unpigmented isolates, obtained from *Zinnia elegans*, were inoculated to plants of lettuce (*Lactuca sativa*), tomato (*Lycopersicum esculentum*), cornflower (*Centaurea cyanus*), godetia (*Godetia grandiflora*), hydrangea (*Hydrangea macrophylla*) and white clover (*Trifolium repens*), reisolations of *A. zinniae* from diseased tissue of these hosts invariably showed intense pigment production in culture. Subsequent colonies grown from such pigmented cultures were generally free from pigmentation.

7. LONGEVITY IN CULTURE.

Studies were conducted to determine the length of time A. zinniae could remain viable and also retain its ability to sporulate on artificial media.

Neergaard (1945) states that only quite fresh isolates sporulate vigorously. The capacity for sporulation is lowered markedly within a few months of isolation and the fungus may completely lose its ability to produce spores as early as 3 or 4 months from isolation. Isolates appear to differ in the duration of their virulence (Neergaard loc. cit.)

The cultures of A. zinniae used in this study varied in age from 13 to 1,820 days. Colonies older than 32 days had been stored on slopes of PDA in sealed test tubes. Plates of PDA were inoculated with a 5mm diameter inoculation piece from the fringe of colonies of each of the 46 cultures used. Two plates were used per culture and the plates held at 28°C.

Colony diameter was recorded after 10 days. Sporulation capacity was determined using the ultraviolet light method.

The results of growth rate and spore production for both chromogenic and non-chromogenic cultures are presented in Table 9 and graphically in Figures 3 and 4.

TABLE 9. GROWTH RATE AND SPORE PRODUCTION OF AGED CULTURES.

Age days	Date of isolation	Source of isolate	Number of cultures		Average colony diameter		Average spore production 1,000s/ml.	
			C	NC	C	NC	C	NC
13	28.12.63	MC	-	2	92	84	102	189
32	9.12.63	SI	2	2	71	75	87	157
88	14.10.63	SC	2	2	69	77	52	127
150	13.8.63	SI	1	2	68	75	28	41
177	17.7.63	SI	2	2	81	80	23	30
198	26.6.63	MC	-	2	-	77	-	68
220	4.6.63	SI	-	2	-	77	-	81
252	3.5.63	SF	2	2	84	76	27	34
262	23.4.63	SI	2	4	87	64	32	40
305	20,3.63	SI	1	2	76	55	18	50
347	6.2.63	MC	2	1	81	38	7	42
368	16.1.63	SF	2	-	84	-	7	-
407	8.11.62	SF	2	2	0	18	0	8
1820	4.1.59	SF	1	2	0	0.53[a]	0	0.20[a]

KEY: [a] One culture non viable, the other produced a colony 53mm in diameter after 10 days.

C = chromogenic culture

NC = non-chromogenic cultures

SF = spores from field infected zinnia plants.

SI = spores from inoculated zinnia plants.

SC = spores from PDA culture.

MC = mycelium from PDA culture.

FIGURE 3. GROWTH RATE OF CHROMOGENIC (C) AND NONCHROMOGENIC (NC) CULTURES

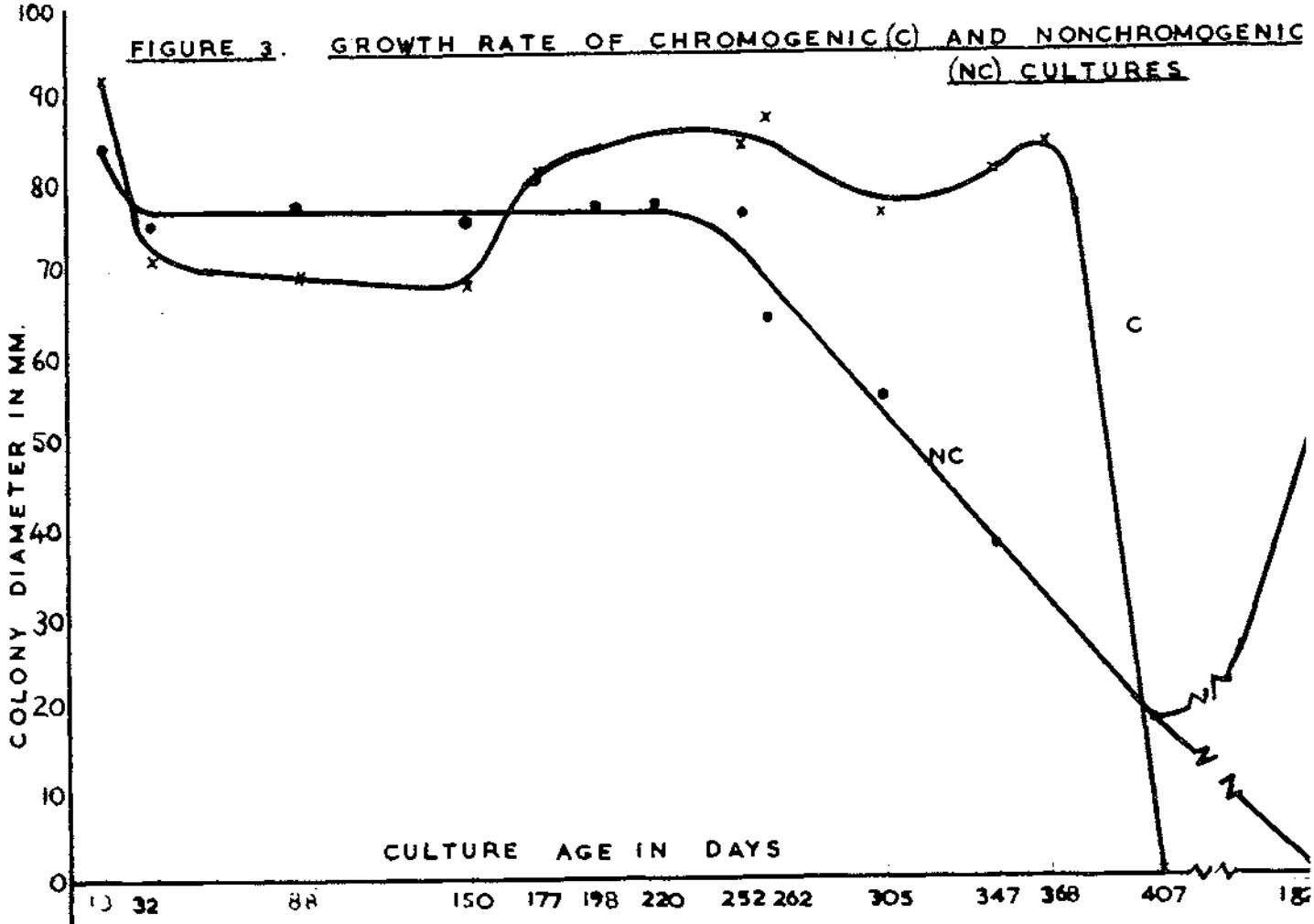
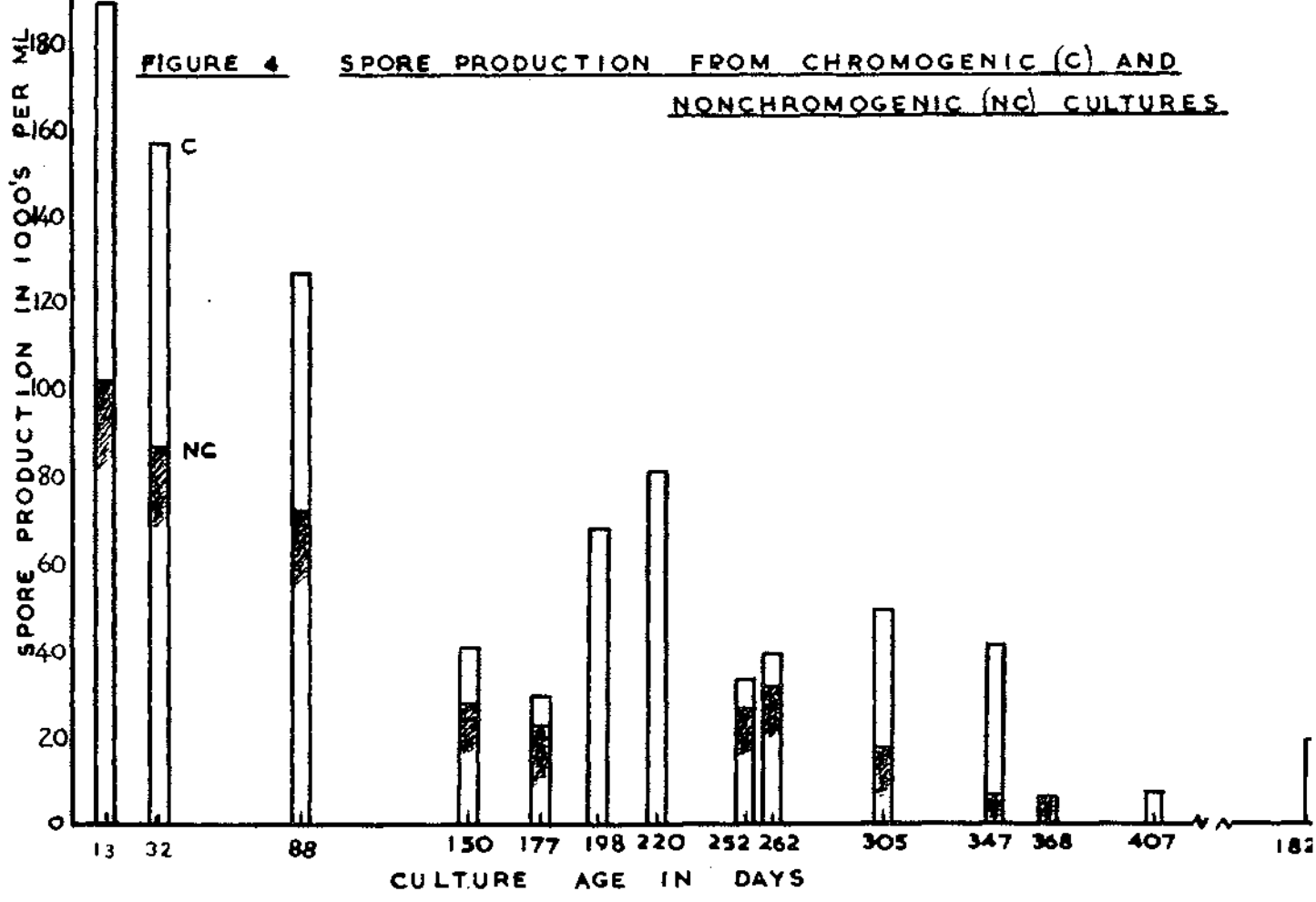


FIGURE 4 SPORE PRODUCTION FROM CHROMOGENIC (C) AND NONCHROMOGENIC (NC) CULTURES



These results indicate that there is a great variation in growth rate and spore production between isolates. However, they do concur with the findings of Neergaard (1945) who noted that, in general, growth rateand sporulation capacity of A zinniae fell off markedly in colonies stored on culture media for more than nine months. After storage for more than 1 year cultural viability, as expressed by growth rate and spore production, was severely impaired.

Chromogenic cultures maintained a higher growth rate than non-chromogenic cultures for up to 1 year on artificial media. Non-pigmented colonies on the other hand, showed a greater capacity for spore production with increasing age in culture than pigmented colonies.

Viability of colonies of A.zinniae, as determined by growth rate and sporulation capacity, was severely depressed after nine months on culture media. As a result, fresh isolations of the fungus from hosttissue were carried out at 3-monthly intervals throughout these studies to maintain colonies which expressed high rates of both growth and sporulation on culture media.

B. PLANT INOCULATION STUDIES.

1. PROOF OF PATHOGENICITY TO ZINNIA PLANTS.

Introduction.

To date there does not seem to be any published work which has proved conclusively that *A. zinniae* is the causal agent of disease symptoms on all parts of plants of *Zinnia elegans*, viz., seedling cotyledons and stems, adult plant roots, leaves, stems and blossoms, and seed.

To determine whether the fungus was capable of attacking and causing disease manifestation on all of these host organs, plant inoculation trials were carried out.

Proof of pathogenicity was obtained using Koch's Postulates.

The morphological characteristics and growth rate of isolates obtained from field infected plants were compared to reisolations of the fungus from plants artificially inoculated with these isolates.

Materials and Methods.

The isolates of the fungus used were obtained from field diseased zinnia plants by transfers of single spores to culture following high humidity treatment of diseased tissue to 28°C for 48 hours (Method 1); or by direct plating of tissue which had been surface sterilised in 0.1% mercuric chloride for 1 minute (Method 2). All isolates were cultured on plates of PDA held at 28°C.

The following isolates were used:-

PLANT PART	HOST VARIETY	METHOD OF ISOLATION.
1. Roots	Giant Dahlia Flowered	2
2. Seedling hypocotyl	Giant Dahlia Flowered	1
3. Seedling cotyledon	Persian Carpet	1
4. Adult plant stem	Lilliput (Rosebud)	2
5. Adult leaf	Giant Dahlia Flowered	1
6. Blossom	Giant Dahlia Flowered	1
7 Seedcoat	Burpee Hybrids	1

After 10 days growth the morphological features and diameter of each isolate was recorded. The ultraviolet light method was used to induce sporulation. Spore concentration was determined by washing conidia from the culture surface with sterile distilled water and recording the concentration of conidia in a total volume of 30ml. using a haemocytometer. The suspension was diluted with sterile distilled water to give a spore concentration from each isolate of approximately 50,000 spores/ml.

The conidial suspensions from each of the seven original isolates were used to inoculate different tissues of healthy zinnia plants. Four plants were inoculated, and two plants used as controls for each isolate treatment.

The method of inoculation varied according to the host organ under study.

(a) Seedling cotyledons, adult leaves and blossoms were atomised with the spore suspension to runoff point using a "Windex" sprayer. Control plants were atomised with sterile distilled water.

(b) Seedling hypocotyls were inoculated using a fine nozzle pipette to place drops of spore suspension on the stem, care being taken to ensure that the tip of the pipette did not scratch the tissue. Controls were inoculated with drops of sterile distilled water.

(c) Stems of adult plants were inoculated by placing 1cm. squares of agar and mycelium in contact with the stems, about 1" above ground level, and securing the inoculum with transparent adhesive tape. Controls were inoculated with agar squares from fresh plates of PDA.

(d) Roots of healthy plants were washed free of soil and dipped in the spore suspension prior to planting in pots of sterilised soil. The roots of healthy plants were dipped in sterile distilled water prior to transplanting as controls.

(e) Seedcoat inoculation was carried out by soaking 50 seeds in the spore suspension in a 250 ml. flask. After 12 hours the seed was tipped onto sterile blotting paper to dry. Fifty seeds were soaked in sterile distilled

water for 12 hours as controls. Unfortunately a completely disease-free seedline was not available for seedcoat inoculation. The seedline used was a line of variety Old Mexico which had given rise to only 0.5% diseased seedlings on blotters in seed screening trials.

Following inoculation, all plants and seeds were placed in a high humidity cabinet for 48 hours and then removed to the glasshouse bench. Disease rating was recorded on a scale 0 to 5, 10 days after inoculation (seven days for inoculated seed). A description of manifest symptoms was also recorded.

Diseased tissue was removed to the laboratory and the fungus reisolated to plates of PDA by the same methods used for obtaining the original isolates, i.e. high humidity treatment or direct plating.

Plates were held at 28°C for 10 days. Colony diameter and morphology were recorded at 10 days.

RESULTS and DISCUSSION.

Results of the severity of attack by the fungus on inoculated plants, and of colony diameter of isolates on culture media are recorded in Table 10

TABLE 10. A COMPARISON OF DISEASE RATING ON INOCULATED PLANTS AND OF COLONY DIAMETER AFTER 10 DAYS ON CULTURE MEDIA.

Source of original isolate.	Colony diam. original isolate.	Inoculum conc. (1000s per ml.)		Plant part inoculated: Root	Seedlg. stem.	Seedlg. leaf.	Adult stem.	Adult leaf	Blossom.	Seed.
ROOT	85	48	D.R.	2	5	5	4	5	5	4
			C.D.	84	86	82	85	84	86	83
			D.M.	-0.3	+1.7	-2.3	+0.7	-0.3	+1.7	-1.3
SEEDLING HYPOCOTYL.	84	52	D.R.	3	3	4	2	4	5	3
			C.D.	82	83	82	80	84	83	87
			D.M.	-1.0	0	-1.0	-3.0	+1.0	0	+4.0
SEEDLING COTYLEDON.	87	50	D.R.	2	2	5	4	5	5	3
			C.D.	85	86	82	89	86	83	85
			D.M.	-0.1	+0.9	-3.1	+3.9	+0.9	-2.1	-0.1
ADULT STEM.	84	49	D.R.	2	3	2	3	4	3	4
			C.D.	82	82	84	83	85	83	84
			D.M.	-1.3	-1.3	+0.7	-0.3	+1.7	-0.3	+0.7
ADULT LEAF.	83	48	D.R.	3	3	5	3	5	5	3
			C.D.	84	82	83	81	83	83	82
			D.M.	+1.4	-0.6	+0.4	-1.6	+0.4	+0.4	-0.6
BLOSSOM.	85	52	D.R.	3	5	5	4	5	5	4
			C.D.	82	86	87	84	86	85	84
			D.M.	-3.0	+1.0	+2.0	-1.0	+1.0	0	-1.0
SEEDCOAT.	81	46	D.R.	2	5	3	2	5	4	4
			C.D.	80	80	83	82	81	82	84
			D.M.	-1.7	-1.7	+1.3	+0.3	-0.6	+0.3	+2.3

KEY: D.R. = Disease rating. 0 = Nil, 1 = Trace, 2 = Slight, 3 = Moderate, 4 = Heavy, 5 = Very heavy.
C.D. = Colony diameter of reisolations on culture media in m.m.
D.M. = Difference from mean colony diameter for all plant parts inoculated in m.m.

These results show that all seven of the isolates used were pathogenic and produced disease symptoms on reciprocally inoculated zinnia plant parts. Similarly, the fungus was associated in every case with the disease, and in no case was disease manifestation present from which the fungus could not be reisolated.

Colony diameters of all isolates were very similar and varied by only 9 mm. (80 to 89 mm.) after 10 days growth at 28°C. This variation is within the range of cultural variability exhibited by different isolates of A. zinniae throughout these studies.

Symptom expression on zinnias following artificial inoculation was macroscopically identical with symptoms from which the original isolates of the fungus had been obtained. A full description of symptom expression by A. zinniae is given in a following section.

The cultural morphology of the 49 reisolates from inoculated plant tissues were macroscopically identical with the cultural characteristics of the original isolates used for host inoculation. In all cases colonies were grey in colour with the fringe of the colony white. Aerial mycelium production was sparse on PDA, being cottony, white and 2-3mm high. Of the original isolates used in this study all were non-chromogenic in culture except the isolates from the seedcoat and from the seedling hypocotyl. The latter both produced a yellow to orange pigment which diffused into the medium, often extending beyond the periphery of the mycelium. Reisolations of these isolates from inoculated host plants were also chromogenic in culture.

CONCLUSION.

From this study it is concluded that A. zinniae is capable of causing disease on the roots, stems, cotyledons, adult leaves, blossoms and seedcoat of inoculated zinnia plants. The fungus was recovered from diseased tissue of inoculated plants and grown on culture media, producing morphological and physiolo gical characteristics identical with those of the isolates with which the plant inoculations were made.

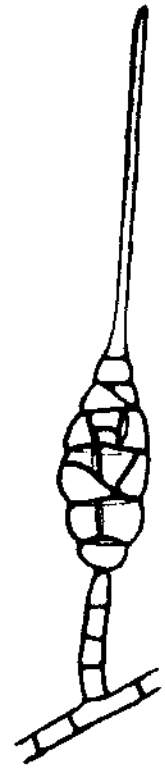
2. SPORE MEASUREMENT STUDIES AND DESCRIPTION OF CONIDIA.

A further proof that A. zinniae is the causal agent of disease symptoms on different organs of Zinnia elegans, conidial morphology and spore measurement studies were carried out to ascertain the variability of conidia from isolates obtained from different tissues of the host.

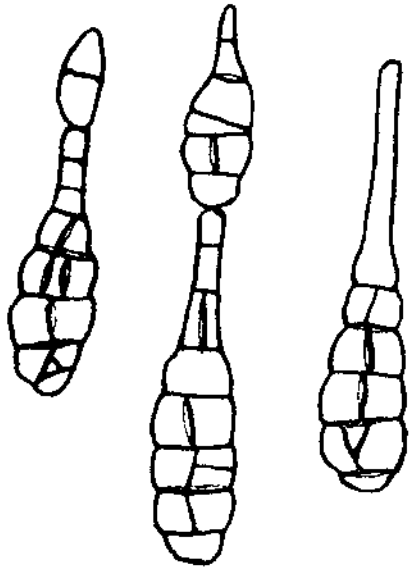
Host tissue from field diseased plants was placed on glass slides in petri plates, the bottoms of which had been lined with moist filter paper. All plates were held at 28°C for 30 hours. This allowed the production of conidia which were all of the same age and which had been produced under identical conditions of temperature and humidity. Conidial morphology was studied under a microscope.

In nearly all cases conidia were borne singly on erect unbranching conidiophores. Rarely, however, two conidia were produced on the same conidiophore, set in a shallow V. Catenulation of spores was seldom seen, but if tissue was allowed to remain at 28°C for up to 10 days, the production of conidia in chai: of 4 to 8 was occasionally observed. The various forms of conidial production are illustrated in Figure 5.

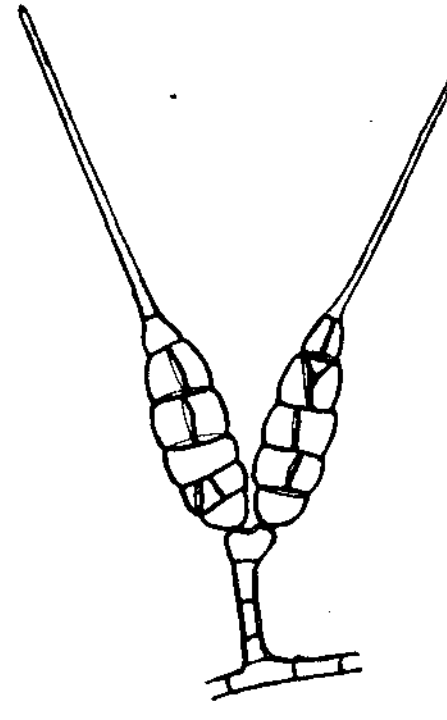
FIGURE 5 FORMS OF CONIDIAL PRODUCTION



SINGLE CONIDIUM
ON CONIDIOPHORE



CATENULATE SPORES
(RARE)



TWO NON-CATENULATE SPORE
ON A SINGLE CONIDIOPHORE
(UNCOMMON)

Conidia from all isolates were large, dark and cylindrical with a terminal beak usually more than twice as long as the body of the spore. In all cases conidia were golden brown in colour when young but became darkly pigmented with age. The spore body was muriform, containing from 3 to 10 transverse septa and 0 to 5 longitudinal septa. A scar at the base of the conidium could often be observed following abscission. The beak of the spore varied considerably in length, even between spores of the same isolate, and contained from 0 to 3 transverse septa.

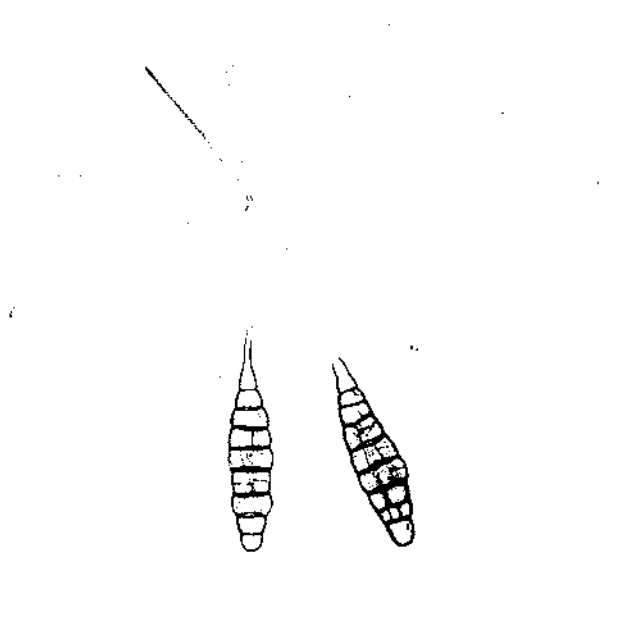


Photo. G. DeLatour.

PLATE 4. Microphotograph of conidia from naturally diseased cotyledons subjected to high humidity treatment, showing spore morphology.

Conidiophores were erect, 1 to 10 septate and brown in colour. They were invariably non-branching with one or more scars, ranging from 4 to 10 u in diameter and up to 100 u in length. On host tissue they were formed singly or in bunches. On agar media they were usually formed singly, either as side

branches on hyphae or as terminal bodies of the hyphae.

To determine whether spore dimensions varied greatly between isolates from different host parts, 50 spores of each isolate were measured on a stage micrometer. The range of conidial measurements obtained from different isolates were compared both with each other and with similar spore measurements of A. zinniae recorded by other workers. Results are presented in Table 11. An analysis of the distribution of spore dimensions within each isolate is recorded in Appendix III.

TABLE 11. COMPARATIVE SPORE DIMENSIONS FROM DISEASED HOST TISSUE (in microns).

Year.	Author(s)	Country.	RANGE IN CONIDIAL MEASUREMENTS. Total length.	Beak length.	Body length.	Body width
1942	Pape	Germany	75-253	N.R.	N.R.	14-27
1943	Dimock & Osborn	U.S.A.	170-210	N.R.	N.R.	20-24
1945	Neergaard	Denmark	39-208.5[a]	6-159	19.5-90	9-28.5
			45-184.5	5-123	30-81	10.5-27
1956	Dingley & Brien	N.Z.	20-64	10-15	20-64	6.5-12
1960	Prota	Italy	N.R.	42-238	36-95	12-29
1961	McDonald & Martens	U.S.A.[b]	36.6-236.4	13.3-146.5	23.3-93.2	8-22
1963	Present study from:-	N.Z.[c]				
	Roots		144-255	94-209	33-64	10-23
	Seedling hypocotyl		117-250	80-193	35-62	12-21
	Seedling cotyledon		103-269	62-207	41-77	16-29
	Adult leaf		123-450	55-377	39-85	12-20
	Blossom		178-316	140-255	33-62	14-23
	Seedcoat		163-350	116-295	39-70	12-23

KEY: N.R. = Not recorded.

[a] = From artificial media.
[b] = From natural material of Sunflower (Helianthus annuus L.)
[c] = Spore dimensions expressed to the nearest whole number.

In the present study the range of conidial dimensions for spores from diseased tissue subjected to high humidity for 30 hours at 28°C were within the same general range for all isolates, i.e. -

Body length	33-85u
Body width	10-29u
Beak length	55-377u
Total length	103-450u.

In general, spore body length and width were the least variable dimensions between isolates. Spore beak length varied over a wide range even between spores of the same isolate. Because of this variability, conidial beak length was considered to be a less reliable criterion for spore comparison than body length.

No direct conclusions can be drawn from a comparison of conidial dimensions recorded in this study and similar measurements by other workers. This is because many variables influence spore size and no other workers appear to record the precise conditions under which spores were produced, nor the age or number of conidia measured.

In particular, five factors influence the value of spore measurements as a diagnostic criterion and as a method of species differentiation.

1. MEDIUM INFLUENCES.

Medium type greatly influences the length of the beak of the spore, but does not appear to cause wide variation in the dimensions of the spore body. On culture media such as PDA, oatmeal agar and cornmeal agar, some of the spores of A. zinniae may lose the characteristic beak. Considerable variation in beak length also occurred between isolates of A. zinniae from different host parts. This effect has also been noted for spores of A. melongenae. (Rangaswami and Sambandam 1960).

2. MATURITY OF THE CONIDIA.

The age of the spores is of great importance in determining the value of spore measurements. The spores of A. zinniae, whether formed on host tissue or artificial media, are subject to change in form and size due to secondary growth.

When first formed the spores are relatively thin, with only shallow constrictions of the body wall at the septa. As the conidia mature beak length increases; body width increases markedly, often to twice the original; the number of transverse septa increases; longitudinal septa become more pronounced and the constriction of the body wall at the septa becomes deeper. The age of spores measured thus markedly affects the range of conidial measurements obtained. The range of dimentions of A. zinniae spores as reported by Dingley & Brien (1956) illustrate this point. The range of variation of both body width and beak length recorded by these workers is far smaller than those recorded in the present study and also by overseas workers, i.e. beak length 10-15u compared with 55-377u, and body width 6.5-12u compared with 10-29u in the present study.

Unfortunately no mention of the age of spores measured is made by Dingley & Brien (loc. cit). It is suggested that the conidia measured by the above workers may have been immature, in that secondary growth had not occurred or was not complete.

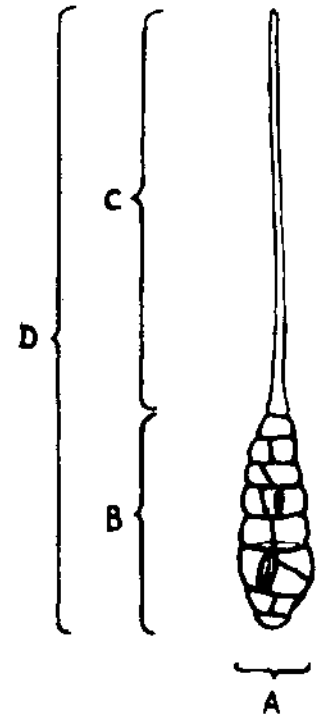
3. NUMBER OF SPORES MEASURED.

The number of spores measured may affect the range of conidial dimensions recorded, due to sampling variations. The number measured should be sufficient to give a fully representative sample.

4. ERRORS IN MEASUREMENT.

Elliott (1917) has stated that considerable variation in spore measurements may occur between workers, and considers that errors in measurement are important in this respect. He conducted a survey of measurement of Alternaria spores by 11 mycologists on the same slide. Dimensions of a certain single conidium varied from 19.2 to 21u X 7 to 10u, a variation of 8.5% X 30%.

In the present study the following dimensions were recorded in spore measurements.



A. Body width
B. Body length
C. Beak length
D. Total length

The point of transition between spore body and beak may vary markedly within the same crop of spores. In the present study the transition point was determined by the terminal septum of the spore body and by judgement of colour changes between the darkly pigmented spore body and the lighter coloured beak.

5. ENVIRONMENTAL CONDITIONS PREVAILING DURING CONIDIAL DEVELOPMENT.

Conditions such as temperature, humidity, light intensity and substrate pH markedly affect spore size. Hence spore size can be used to characterise species only if environmental conditions are completely standardised (Williams 1959).

CONCLUSION.

Conidial morphology and measurements of spores produced under standardised conditions were similar for all isolates of A. zinniae from different host parts. This supports the conclusions of the previous experiment that A. zinniae is capable of producing disease symptoms on different zinnia plant parts.

Hence A. zinniae is considered to be pathogenic to all parts of zinnia plants in all stages of development.

C. TAXONOMY AND NOMENCLATURE OF ALTERNARIA ZINNIAE.

1. TAXONOMY AND FEATURES OF THE GENUS ALTERNARIA.

The genus Alternaria belongs to the family Dematiaceae, order Moniliales of the Fungi Imperfecti. The delimitation of the genus has long given mycologists a number of problems regarding species identification and nomenclature.

Alternaria was first described in 1817 by Nees with A. tenuis as the only species (Neergaard 1945). Since that time such a large number of other species have been described that the genus may now be broken down into the following broad divisions, as described by Neergaard in 1945.

1. Species forming chains of conidia with a short or relatively short beak - e.g. A. tenuis.

2. Species which never form chains of conidia, the spores being sarciniform without any beak, most often with a median major cross-wall accompanied by a constriction, and usually described under Macrosporium, e.g. M. sarcinula.

3. Species forming chains only rarely, the spores being provided with a long filiform beak; sometimes considered as Alternaria, sometimes as Macrosporium, e.g. A. (M) solani, A. zinniae.

Fries (Neergaard 1945) did not recognise the genus Alternaria in his Systema Mycologicum of 1832. Hence, according to the International Rules of Botanical Nomenclature the name is incorrect; and, besides, the original type specimen (A. tenuis) described by Nees has not subsequently been identified. On the other hand, the description given by Nees (Neergaard 1945) is sufficiently thorough to establish that the current concept of the genus Alternaria is in agreement with this, the oldest description of the genus.

Macrosporium is the strictly correct name, being described by Fries in the Systema (Neergaard 1945). Unfortunately this name has been consistently, but erroneously applied to species forming no chains of conidia (group 2 and partly group 3 above.)

Accordingly, Wiltshire (1933) suggests the relegation of the name Macrosporium to the "nomina ambigua", and the use of the nomenclature Alternaria, the older of the two names, for the genus.

Members of the genus Alternaria occur universally. Some species are found as saprobes on dying and dead plants and in the soil. Others are parasitic on plants; e.g. A. solani, causal agent of early blight of potatoes, and A. dianthi, A. dauci and A. zinniae, causal agents of blights on carnation (Dianthus spp), carrot (Daucus carota) and zinnia (Zinnia elegans) respectively. The general mycological features of the genus include -

1. Well developed, hyaline, septate mycelium.

2. Conidia produced on conidiophores which are not enclosed in any form of fruiting body.

3. Large, multicellular conidia with both transverse and longitudinal septa occurring typically. The conidia are usually borne in chains, but not infrequently they are formed singly on the tips of the hyphae which bear them (Alexopoulos 1962). Sporulation, as in all Deuteromycetes is strictly an asexual process.

4. Conidiophores indistinguishable from the somatic hyphae.

2. TAXONOMY OF THE PATHOGEN.

The first record of the fungus causing leafspot of zinnias was by Rostrup in 1902 in Denmark. Rostrup realised that he was dealing with a species not previously described and recorded the causal agent as Macrosporium zinniae n. sp. (Neergaard 1945).

Apparently Rostrup did not publish a description of this fungus. However, in 1923 Gram & Rostrup (Neergaard 1945) recorded an attack on young zinnia plants which they determined to be due to Macrosporium caudatum, Cook & Ellis.

In 1934 specimens of an undescribed leafspot of Zinnia elegans were deposited in the herbarium at Cornell University, New York, by Dearness (Dimock & Osborn 1943 These specimens had been collected from Ontario, Canada, and the organism associate with the spots was tentatively referred to as Alternaria solani (Ell & Mart.) Jones & Grout, although no detailed study of the disease or the pathogen was made at that time.

The first controlled experiment on the pathogenicity of the Alternaria strain involved was carried out in 1934 by Guterman (Dimock & Osborn 1943). Unfortunately no account of this work was ever published. During the years 1936-1940, a zinnia disease apparently identical with that described in 1923 by Gram & Rostrup (Neergaard 1945) was reported by Weber (1937) and Neergaard (1937) in Denmark, and by Beaumont & Staniland (1939) in England. The latter authors described an attack on zinnia seedlings by an Alternaria species, which they took to be identical with Macrosporium caudatum, presumably at the suggestion of Gram & Rostrup's above mentioned determination of a similar attack (Neergaard 1945).

In 1942 Pape observed in Germany the development of an Alternaria disease of zinnia (mainly Z. elegans and Z. haageana). The causal agent responsible for the disease did not appear to agree with the species previously recorded on the same host by other workers. The fungus in question was accordingly regarded by Pape as a new species and designated Alternaria zinniae n. sp.

Neergaard (1945) records that A. zinniae is very closely related to A. tomato (Cke) and questions whether the former ought not to be classified under the latter.

He states that "on the basis of the present description, no particular morphological difference appears to be demonstrable between the two species. Still, the question of whether A. zinniae ought to be maintained as a species per se has to be left unsettled until comparison can be made between indisputably identified sporulating isolates of both species". No further published work has been carried out on this point.

The earliest legitimate name must be adopted as the true nomenclature of fungi according to the International Code of Nomenclature Art. II. (Edmunds & Hanson 1960). Accordingly, the valid nomenclature for the fungus is considered to be Alternaria zinniae, Pape.

A. zinniae Pape, named as such, appears to have been universally accepted as the fungal species causing leafspot of zinnia, and no other record of confusion of A. zinniae with M. caudatum, A. solani or A. tomato has subsequently occurred in th literature.

According to Neergaard (1945), reports of the fungus causing leafspot of zinnia and designated as M. caudatum in 1923 by Gram & Rostrup (Neergaard 1945) and in 1939 by Beaumont & Staniland were "no doubt" caused by A. zinniae. Similarly Neergaard (loc. cit.), on examination of Rostrup's herbarium material in Copenhagen, came to the conclusion that the fungus designated as M. zinniae n.sp was identical with A. zinniae Pape.

C H A P T E R II.

T H E D I S E A S E.

CHAPTER II. THE DISEASE.

A. THE DISEASE AND ITS EFFECTS ON THE HOST.

1. Geographical distribution of the disease.

The geographical distribution of Alternaria disease of zinnias can best be visualised by considering published records of its occurrence. To date these are all from countries in tropical and temperate zones.

In the United States of America the disease has been recorded on zinnias in at least 10 states, viz., California (Baker & Davis 1950), Colorado (Forsberg 1946), Connecticut (Dimock & Osborn 1943), Florida (Weber 1925), New Jersey (Wescott 1950), New York (Dimock & Osborn 1943), Ohio (Ellett 1957), Oregon (Shaw 1958), Pennslyvania (Wescott 1950), and South Carolina (Wescott loc. cit.).

There have been five reports of the disease, on zinnias, from Canada, viz., British Columbia (Jones 1948), New Brunswick (Orlob 1960), Nova Scotia (Conners & Savile 1952), Ontario (Creelman 1952, Kemp 1953).

In Europe the disease has been recorded from Austria (Schmidt 1953), Denmark (Neergaard 1937, De Tempe 1959), England (Beaumont & Staniland 1939), France (Neergaard 1945), Germany (Pape 1942), Holland (Neergaard 1945), Hungary (Neergaard 1945), Italy and Sardinia (Neergaard 1945, Prota 1960) and Yugoslavia (Kispatic 1951, Perisic 1952).

The disease has been reported in Africa from Egypt (Assawah & Elarosi 1959), Kenya (Nattrass 1948), Mauritius (Plant Pathology Division 1959), Northern Rhodesia (Riley 1956), Nyasaland (Corbett 1962), Southern Rhodesia (Hopkins 1950, Bates 1963), and Tanganyika (Wallace & Wallace 1948).

The only records in Asia and the Pacific area have been from India (Kanjansoon & Mathur 1962), New Zealand (Dingley & Brien 1956), and Tasmania (Wade et al. 1959).

Records of the disease on hosts other than zinnia will be reviewed in the section on Host Range.

2. Description of the disease and its effects on the host.

Alternaria disease (Alternariosis) of zinnias is characterised by its effects on both seedlings and adult plants. Descriptions of symptoms of the disease have been made by a number of workers over the past 25 years and these accounts sh a high degree of uniformity (Pape 1942, Dimock & Osborn 1943, Neergaard 1945, Forsberg 1946, Baker & Davis 1950, Dingley & Brien 1956, Prota 1960). Although most authors have described symptoms of leaf, stem and blossom attack on adult plants, few have fully described disease manifestation on seedlings or roots of Zinnia elegans.

In New Zealand, all zinnia seed sown by commercial growers is imported, mainly from U.S.A. and Europe. The majority of this imported seed is sown by nurserymen and the seedlings raised as bedding plants for public sale. Since no visual symptoms of disease can be observed by macroscopic examination of the seed, the first evidence that a particular seedline is diseased is symptom expression on the developing seedlings.

Seed is sown in boxes of sterilised soil under glass. The seedboxes are watered liberally and sown to give dense stands of seedlings, factors which no doubt provide environmental conditions of high humidity within the seedboxes, conducive to the development and spread of the disease.

Some seedlings rot completely and fail to emerge, while others bear dark lesions on the cotyledons and stems and may damp-off after emergence. On seedlings, the first evidence of the disease is the presence of lesions on the expanding cotyledons, which emerge in about 7 to 10 days. The seedcoat is invariably borne above soil level, adhering to the cotyledons. Cotyledonary lesions are often evident when the testa falls to the ground. In some cases the testa may remain attached to one of the cotyledons for up to two weeks after emergence and lesions may occasionally be seen growing out from the seed-coat into seedling leaf tissue.

PLATE 5. COTYLEDONS SHOWING LESION ORIGINATING FROM THE ADHERING SEEDCOAT.

Lesions on the cotyledons are dark brown to black in colour. Initially they may be localised and irregular in outline, but often enlarge to cover the entire leaf surface. Older lesions may show a "target board" effect involving concentric ridging due to mesophyll collapse within the leaf. Diseased tissue shrivels and the cotyledons, if severely infected, collapse and die. The cotyledonary phase of the disease is illustrated in Plate 6.

PLATE 6. LESIONED COTYLEDONS FROM PLANTS ARISING FROM DISEASED SEED. TEN DAYS FROM SOWING.

Lesions due to secondary spread of the pathogen from initial cotyledonary lesions generally appear from about 14 to 16 days after emergence.

Stem attack usually becomes evident at about the same time as secondary lesions occur on the cotyledons. Stem lesions are manifest as watery necrotic zones of decay at, or slightly above, ground level. In some cases the lesions may appear on the hypocotyl directly below the cotyledons. In only rare instances is stem lesioning seen on seedlings which do not show cotyledon infection. The soft, succulent tissue of the seedling hypocotyl appears to be extremely susceptible to attack and in many cases rotting may be so severe as to completely girdle the stems and cause plant collapse. Stem sttack is thus often manifest as a post-emergence damping-off. The lesions are dark brown to black in colour, with sunken centres, and are common on the stems of seedlings in infected plantings.

PHOTO. H. DRAKE.

PLATE 7. DISEASED SEEDLINGS SHOWING COTYLEDONARY LESIONS AND STEM DECAY.

The roots of infected seedlings may remain healthy despite the presence of both leaf and stem attack, but in some few cases plant wilting due to root decay may occur. Wilted plants are easily pulled from the soil and the roots show rotting and decay. Such rotted zones are easily broken off and the cortical tissues may slough off completely. Root attack, however, is generally considered to be more important in adult plants.

As the seedlings develop, secondary spread of the pathogen becomes evident as localised lesions on the expanding leaves. This lesioning gives the plants an unsightly appearance, often rendering them unfit for sale.

Photo. H. Drake.

PLATE 8. COMMERCIAL SEEDLINGS WITH LEAF LESIONING.

Seedlings are usually transplanted when about three weeks old. During the transplanting process any seedlings showing disease symptoms are discarded. Some plants which appear healthy at transplanting may subsequently show disease manifestation. In some seedlings the pathogen has infected, but requires an incubation phase of one to two days before disease symptoms appear.

Plants are generally sold to the public when 5 to 6 weeks old. Many plants are killed outright before sale due to leaf and/or stem attack while others escape the seedling phase of the disease.

In adult plantings, which are invariably made outdoors, many plants show severe development of the disease following wet, misty weather or heavy dews. In some cases adult leaves may be so severely diseased that they wilt and die, giving the plants a scorched appearance.

On leaves of adult plants the disease appears on both leaf surfaces as

irregularly scattered, dark brown to black lesions, o.5 to 1.5cm. in diameter. On the upper leaf surface the lesions often show a characteristic whitish-grey patch approximately 1mm in diameter within the lesion.

PLATE 9. LESION ON A NATURALLY DISEASED LEAF (X6).

The lower, older leaves are the first to contract infection which, in severe cases, may extend to the young foliage nearer the top of the plant.

Older lesions may develop a reddish margin and are dark brown with a somewhat lighter coloured centre.

Photo. H. Drake.

PLATE 10. LEAVES OF VARIETY PERSIAN CARPET SHOWING ADULT LEAF-SPOT PHASE OF THE DISEASE.

Differentiation of margin and centre is lacking on the lower leaf surface. On the upper leaf surface a "target board" effect may be evident, the lesions showing concentric ridging due to mesophyll collapse. In severe attacks the spots may become confluent and diseased leaves may wither completely. Dead tissue is very fragile and disintegrates easily. This gives diseased leaves a very ragged appearance.

PLATE 11. ADULT LEAF SHOWING FRAGMENTATION OF DISEASED TISSUE.

Stems of adult plants may also be attacked, the lesions appearing as black, elongate, longitudinal streaks. When numerous, such lesions may coalesce. These lesions generally occur on the internodes of the stem and may be superficial. Larger lesions, which frequently girdle the stem, often originate from the nodes, due either to growth of the fungus inwards from infected leaves or to infection occurring directly in the leaf axils. Unlike the internodal spots, the nodal lesions ordinarily do not remain superficial, and distal portions of affected stems may be killed by complete girdling at the nodes. Stem tissues are often invaded by growth of the fungus from badly infected leaves. Basal cankers are also common on the stems of plants in infected beds. Affected plants often wilt completely, even when the lesions do not entirely girdle the stem. The root systems of some plants showing stem decay may remain healthy.

Root infection, while the least conspicuous, is perhaps the most serious phase of the disease since it is not amenable to control by the measures that might be employed to control infection on stems, foliage and blossoms (Dimock & Osborn 1943).

Root attack is manifest by the outer tissues of affected roots becoming dark grey to black in colour. These roots rot completely and the cortical tissues slough off, resulting in wilting and death of plants in severe cases. Plants suffering from severe root attack are prone to collapse in high winds and are easily pulled from the soil.

In some cases the severity of stem attack may be related to the amount of root decay. Plants showing severe stem lesioning often have heavily diseased roots. Conversely, plants with only light stem infection may often have macroscopically disease-free root systems.

PLATE 12. Plants showing varying amounts of stem and root attack. Plant on left severely diseased, with heavy stem decay and roots almost completely rotted. Plant on right showing slight stem lesioning and macroscopically disease-free root system.

Root damage results in wilting due to reduced water absorbing capacity by the plant. Plants with diseased root systems often partially recover at night, but show severe wilting during hot weather.

Blossom lesions may become evident on the conspicuous ray florets. These spots may be up to 1 cm. in diameter and are either circular, or occur as longitudinal streaks. Lesions are dark brown in colour. Infected petals soon darken and wither, causing the blossoms to become very unsightly and rendering them worthless as cut flowers or garden specimens.

PLATE 13. Diseased blossom, variety Giant Dahlia flowered, showing lesions as longitudinal streaks on the ray florets.

CONCLUSION.

The symptoms of Alternariosis on *Zinnia elegans*, as described in this study, appear to be very similar to those recorded by various overseas workers. This indicates that there is a high degree of uniformity of symptom expression of the disease in different countries. In New Zealand the seedling phase of the disease appears to be of considerable importance. In most cases this aspect of the disease is not noted as being of any consequence in other countries. Root attack, because it is not generally noticed in outdoor plantings, is also not considered to be important overseas. In New Zealand, however, this phase of the disease can be quite serious, especially in beds planted with zinnias in successive seasons.

B. THE DISEASE CYCLE.

In general, the following stages can be recognised in the development of a plant disease where a fungus is the causal agent;

1. Spore germination.
2. Penetration.
3. Infection of the host.
4. Incubation.
5. Manifestation.
6. Exit from the host and secondary spread.
7. Overwintering.

These stages collectively make up the "Disease Cycle" and are considered as separate entities in this chapter although they may be integrated in the life cycle of the pathogen.

1. Spore germination.

Spore germination is the initial stage in the development of fungal mycelium from its spore. It differs from the growth of hyphae primarily in the ability of a spore to utilise its stored reserves for metabolism (Gottlieb 1950). Germination of the spore may be considered the first step in the development of the Disease Cycle. Those factors affecting spore germination are considered to be of importance, since an understanding of them could indicate the environmental conditions under which germination of the spore is most likely to occur. It is generally considered that a single fungal spore is capable of germinating and establishing a successful infection (Garrett 1960). Accordingly studies were carried out to determine the environmental conditions which affect the germination of individual conidia of *A.zinniae*.

A study of the literature on spore germination as it is affected by environmental factors revealed that temperature and humidity were considered to be the two most important factors involved (Tomkins 1932, Hawker 1950, Lilly & Barnett

1951, Cochrane 1960, Goos 1962). Accordingly studies were undertaken to ascertain the effect of these factors on the germination of conidia of A. zinniae.

Two variables which have caused considerable disparity in the results of spore germination between workers are the criterion for measurement of spore germination, and opinion as to when, in fact, a spore has begun to germinate.

Various criteria have been used to determine the effect of different physiological agents on germination. Of these the most frequently used is the final germination of the population. Other standards of measurement are the time required for germination (the latent period of germination), the length and rate of elongation of the germ tubes (Cochrane 1960), and the percentage number of spores germinated after some arbitrary time (Tomkins 1932).

Similarly, opinions differ as to when a spore may be considered to have germinated. For some workers this is the moment when a swelling in the spore wall appears (Gottlieb 1950, Hawker 1950); for others it is the time at which the germ tube has parallel walls (Gottlieb 1950, Bollard 1950); while a third group use the time when the germ tube length equals the width or length of the spo (Gottlieb 1950).

Tomkins (1932) considers that in any comparison of spores germinated under conditions which differ in respect to one variable only (e.g. temperature, moisture) in order to find the effect of the variable on germination, the latent period and the rate of elongation are of most importance. He considers that only by recording those two factors, along with the percentage of spores germinating can the course of germination be fully described.

In the present study spores were considered to have germinated when the length of the germ tube equalled the width of the spore body - approximately 20 u. This criterion was chosen because it was noted in preliminary trials that at temperature extremes, i.e. above 40°C and below 5°C, spores produced germ tubes which elongated to about 5-10 u and then failed to continue growth.

Measurement of spore germination was determined by considering the time for germination of definite percentages of spores as suggested by Bonner (1948), the

average length of the germ tubes produced by the spores, the average growth rate of the germ tubes in relation to time and the total percentage germination of the population.

Morphologically, three distinct events characterise the process of spore germination in the asexual spores of the higher fungi (Cochrane 1960),

1. Nuclear division at some time during the process.
2. Imbibition causing spore swelling.
3. Germ tube appearance.

Germ tube growth presumably follows the same course as mycelial growth, extension occurring only at the tip (Cochrane 1960).

In cases where the percentage to be recorded did not coincide with an observation period, the time required for the germination of a definite percentage of spores was extrapolated graphically.

(a) The effect of temperature on spore germination.

The effect of temperature on germination was ascertained by observing and counting the percentage germination at frequent intervals. In all cases 100 spores per slide were considered, three slides being used for each treatment. Using this method very little variation on different slides was noted in germ tube development and germination percentage in relation to time.

To determine which substrate was best suited for the germination of spores of A. zinniae, several different nutrient and non-nutrient substrates were compared. It was felt that laboratory studies would provide information of practical value only if they were conducted under conditions which were likely to occur in nature. Tomkins (1932) states that the percentage of spores that germinate in any given sample is largely determined by the nutrient solution. Some spores do not germinate in water, some do so freely, while others behave irregularly. Thus Tomkins (1932) considers that nutrient solutions should be used for germination tests wherever possible.

To try to obtain a substrate on which spore germination occurred in a

manner similar to that occurring in the field the following types of substrate were compared:-

1. Drops of tap water on glass slides.
2. Drops of sterile distilled water on glass slides.
3. Drops of sterile distilled water in hanging drop slides.
4. Squares of tissue (1cm) cut from the leaves of 6-week-old zinnia plants and set out on glass slides.
5. Thin films of zinnia leaf agar on glass slides.
6. Thin films of PDA on glass slides.

All slides were placed in petri plates which had been lined with moistened filter paper. All plates were held at 28°C for 2 hours prior to substrate inoculation with spores. In each test the inoculum used was a suspension (6000 spores/ml) containing spores produced from 10-day-old cultures grown on PDA.

Each substrate was inoculated with a drop of the spore suspension and the plates held at 28°C.

Counts of percentage germination and observation of germ tube development were made after 4 hours, three lots of 100 spores being observed for each substrate. The results are recorded in Table 12:

TABLE 12. COMPARISON OF SUBSTRATES FOR SPORE GERMINATION STUDIES.

Substrate	% Germination Average ± SE.	Maximum no. Germ Tubes per Spore.	Range in Length Germ Tubes (Spore diameters)	Substrate pH.
Tap water glass slide	86.5±1.5	9	1-5	7.5
Sterile water glass slide	80.0±1.1	8	1-6	7.1
Sterile water hanging drop slide	82.0±0.6	5	1-5	7.1
Zinnia leaf tissue	87.3±1.7	10	1-7	6.8-6.9
Zinnia leaf agar film	87.7±1.0	12	1-8	6.9
PDA film	88.0±0.8	10	1.5 -9	5.8

Germination criterion - when germ tube length equalled width of spore body.

These results show that conidia of A. zinniae are capable of germination in sterile distilled water. This indicates that no external nutrients are necessary and water as a substrate appears to be quite satisfactory for spore germination. All of the substrates studied gave similar results as measured by average germination percentage and germ tube development after 4 hours at 28°C.

It thus appeared that the substrate that could be handled with the greatest ease was the most desirable. The reasons for accepting or discarding various substrates as suitable for spore germination studies are presented below.

1. Water on glass slide.

   Advantage - Ease of preparation.

   Disadvantages - Care is required in handling slides to prevent water drops running together and altering spore position on the slides. Need for water replenishment at frequent intervals, especially at high temperatures, due to evaporation.

2. Water in hanging drop slide.

   Disadvantages - Time factor in preparation of large numbers of slides. Difficulty in location of individual spores. Congregation of spores in a mass at the bottom of the drop. Problems of water replenishment following evaporation at high temperatures.

3. Zinnia leaf tissue on glass slide.

   Advantage - Observations of germination closely approximate field conditions.

   Disadvantages - Opacity of leaf prevents easy location of spores and germ tube measurement. Germ tubes hard to detect against leaf background.

4. Agar film on glass slide.

   Advantages - Provides a solid substrate. No problem of spore movement. Provides a clear substrate, especially if agar is filtered before use.
   The spore suspension forms a thin film on the agar and the spores are left adhering to the agar when the water evaporates.
   Provides percentage germination figures similar to those on zinnia leaf tissue.

In view of these factors, the method using an agar film on a glass slide was considered to be the most practical.

To determine whether the pH of the agar medium would be a limiting factor affecting germination, spores were placed on PDA and zinnia leaf agar films with a pH range of pH2 to pH10. The glass slides bearing inoculated agar films were placed in petri plates lined with moistened filter paper and held at 28°C. After 6 hours, germination percentage counts for each pH value were made in each of three random low power fields.

The results, expressed as an average percentage germination are recorded in Table 13:

TABLE 13. SPORE GERMINATION ON AGAR FILMS ON VARYING pH VALUES.

pH of Agar.	Percentage Germination Spores After 6 hours at 28°C Zinnia Leaf Agar.	PDA.
2	12	5
3	22	11
4	27	18
5	84	93
6	96	96
7	96	98
8	97	92
9	96	97
10	95	96

Normal pH. PDA 5.8
Zinnia leaf agar 6.8 to 6.9

These results indicate that at pH values between 5 and 10 the hydrogen ion concentration is not a limiting factor to germination of conidia of A. zinniae That pH is not important in depressing spore germination in nature is shown by the fact that sterile distilled water allowed to lie on the surface of a zinnia leaf for 24 hours had a pH 6.8 - 6.9, and rain water collected from the surface of zinnia leaves in the field had a pH ranging from 6.0 to 6.6. These figures fall within the range of pH values at which the spores of A. zinniae will readily germinate.

Of the two agar substrates, PDA was finally chosen for use in further studies because of its ease of preparation and the ready availability of its components.

The method used to determine the effect of temperature on spore germination of A. zinniae is detailed below:

Clean glass slides (75 x 25mm) were placed in petri plates lined with moistened filter paper. Liquid PDA, which had been filtered through a blotting-paper-mache filter twice, was placed on each slide. Approximately 3ml of PDA was placed on each slide and spread evenly over the surface with a sterile wire loop. The resultant film was allowed to solidify at room temperature. A series of petri plates containing slides with agar films was placed in incubators at each of the required temperatures for 1 hour prior to inoculation to allow the substrate temperature to equilibrate with that of the incubator.

Spores of A. zinniae were produced on scraped 10-day-old cultures on PDA. The spores were washed from the culture surface with distilled water and the spore suspension thus obtained (approximately 4000 spores per ml) was held in a refrigerator at $2^{o}C$ if not required immediately.

The petri plates were removed from each incubator and the agar film on each slide inoculated by running approximately 1ml of the spore suspension along the length of the film, using a sterile 60s nozzle dropper. This provided a uniform layer of spores on the agar surface. The petri plates were then immediately replaced in the incubator from which they had been removed.

Relatively low concentrations of spores were used in these studies to overcome any self-inhibition effect on germination, which may take place when spores occur together in large numbers. By this method it was hoped to obviate the effects of toxins produced by a mass of spores, which could reduce the germination percentage and give a false picture of the potential of conidia for germination.

It was unfortunately impossible to observe all temperature series concurrently due to limitations of time and incubator space. The study was therefore conducted in three parts.

1. Effect of temperatures of -5, + 1, 5, 10, and 17°C on spore germination.

2. Temperatures of 20, 22, 24, 26 and 28°C.

3. Temperatures of 30, 33, 35, 37, 40 and 45°C.

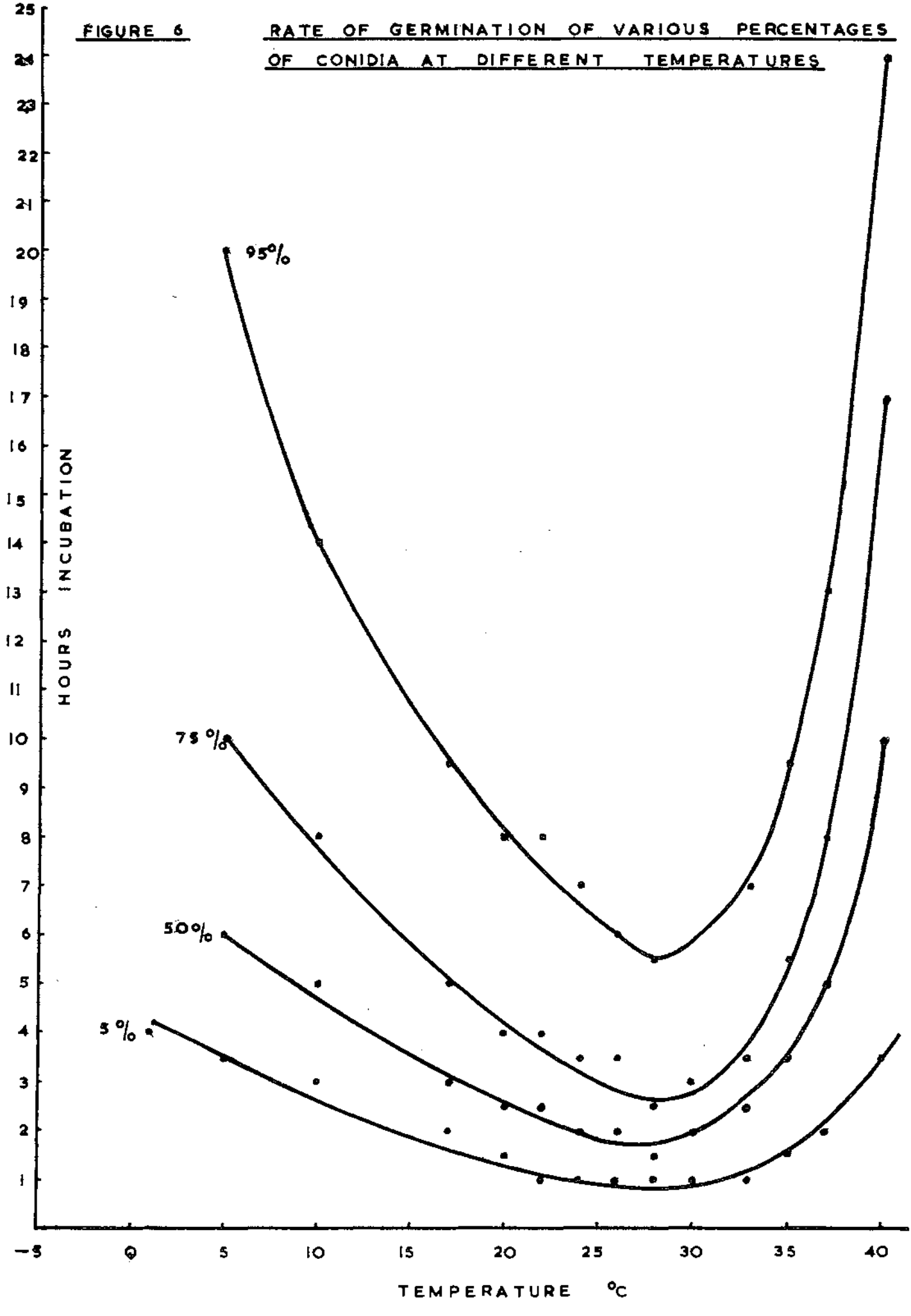
Observations were made at intervals of ½ hour during the early stages of germination and later at longer intervals as determined by spore germination rate. With frequent observations it was not possible to use the same slides repeatedly because exposure to room temperature during the measuring process would tend to alter the spore germination rate. Consequently a set of slides was prepared for incubation at each temperature and after each measurement the particular slide used was discarded.

A "Veeder" hand counter was used to record the number of spores germinated in each of three random low power fields. One hundred spores were observed in each field and the number which had germinated was expressed as a percentage of the total number of spores observed. The trial at each temperature was discontinued when an average germination of 95% or over was obtained.

The time taken to reach 5, 50, 75 and 95% germination was noted and these times are recorded in Figure 6. Percentage germination figures at each temperature are presented in Appendix IV. The development of the germ tubes as shown by the maximum number of tubes produced per spore, and the maximum length of the germ tubes of spores incubated at each temperature is shown graphically in Figure 7.

At temperatures of 1°C and 45°C a number of spores initiated germ tubes which failed to elongate more than 5 - 10 u in length. After 48 hours incubation at these temperatures slides were transferred to an incubator at 28°C. The germ tubes of spores which had been held at 45°C failed to recommence growth. The spores which had been incubated for 48 hours at 1°C and which had initiated germ tubes at this temperature showed rapid germ tube elongation when subsequently placed at 28°C. Because the length of germ tubes produced at 1°C and 45°C on agar films were not as long as the width of the spore body they were not recorded in the germination percentage. If the criterion of spore germination had been

FIGURE 6 RATE OF GERMINATION OF VARIOUS PERCENTAGES OF CONIDIA AT DIFFERENT TEMPERATURES



that spores were considered to have germinated when the sides of the germ tube became parallel, a number of spores held at 1°C and 45°C would have been credited as having germinated, viz., 7% of spores held at 1°C and 6% of spores at 45°C, both after 12 hours.

From the results shown in Figure 6 the cardinal temperatures for spore germination of A. zinniae on PDA films were below 5°C, 28°C and above 40°C.

Figure 6 shows that temperatures above the optimum of 28°C had a far greater effect in depressing the germination of spores of A. zinniae than did corresponding temperatures below 28°C.

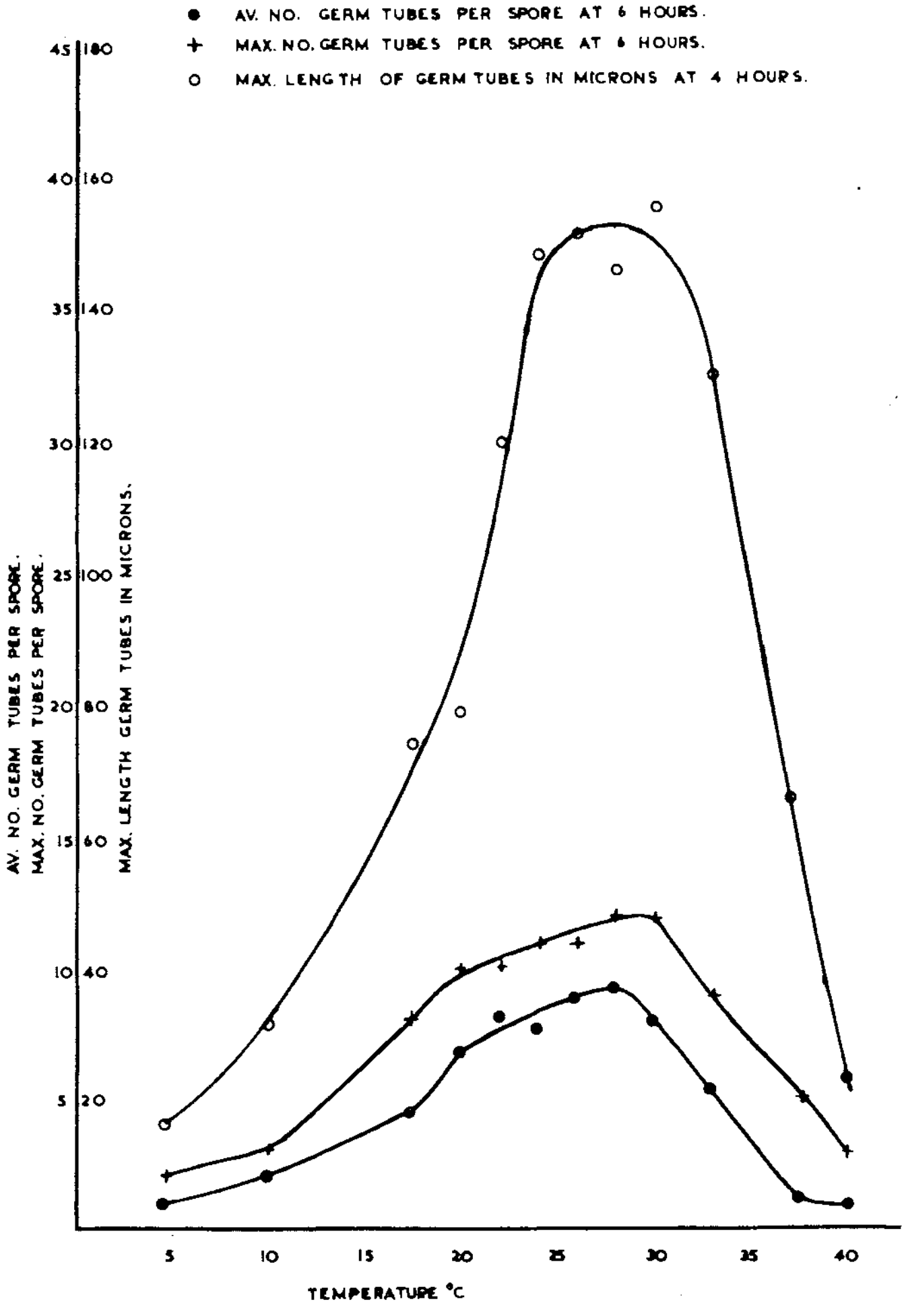
The time taken for 75% germination at 28°C was 2.3 hours and a further 3.2 hours were required for an additional 20% of the spores to germinate. This may indicate that there is some antagonistic or inhibitory material produced by germinated spores which retards the germination of others nearby. This trend was consistent to all temperatures.

If it is conceded that leaf moisture is essential for germination of spores in nature, moisture must be present on the leaf for longer periods when the temperature is markedly higher or lower than the optimum of 28°C to allow successful germination of large numbers of spores. Presuming that all germinated spores were capable of successfully establishing a disease relationship with the host, the length of time that moisture remained on the leaves of zinnia plants could have a marked effect on the number of infections, as evidenced by spore germination results in this study.

Germ tube number and length increased with temperature of incubation from 5°C up to 24-30°C and then progressively declined between 33 and 40°C as shown in Figure 7.

Germ tubes were first produced from the basal cell of the conidium, but in most cases this was followed by rapid production of germ tubes from other cells in the spore body. The beak or "tail" of the conidium of A. zinniae is generally considered to be sterile in that it does not produce germ tubes. In the present

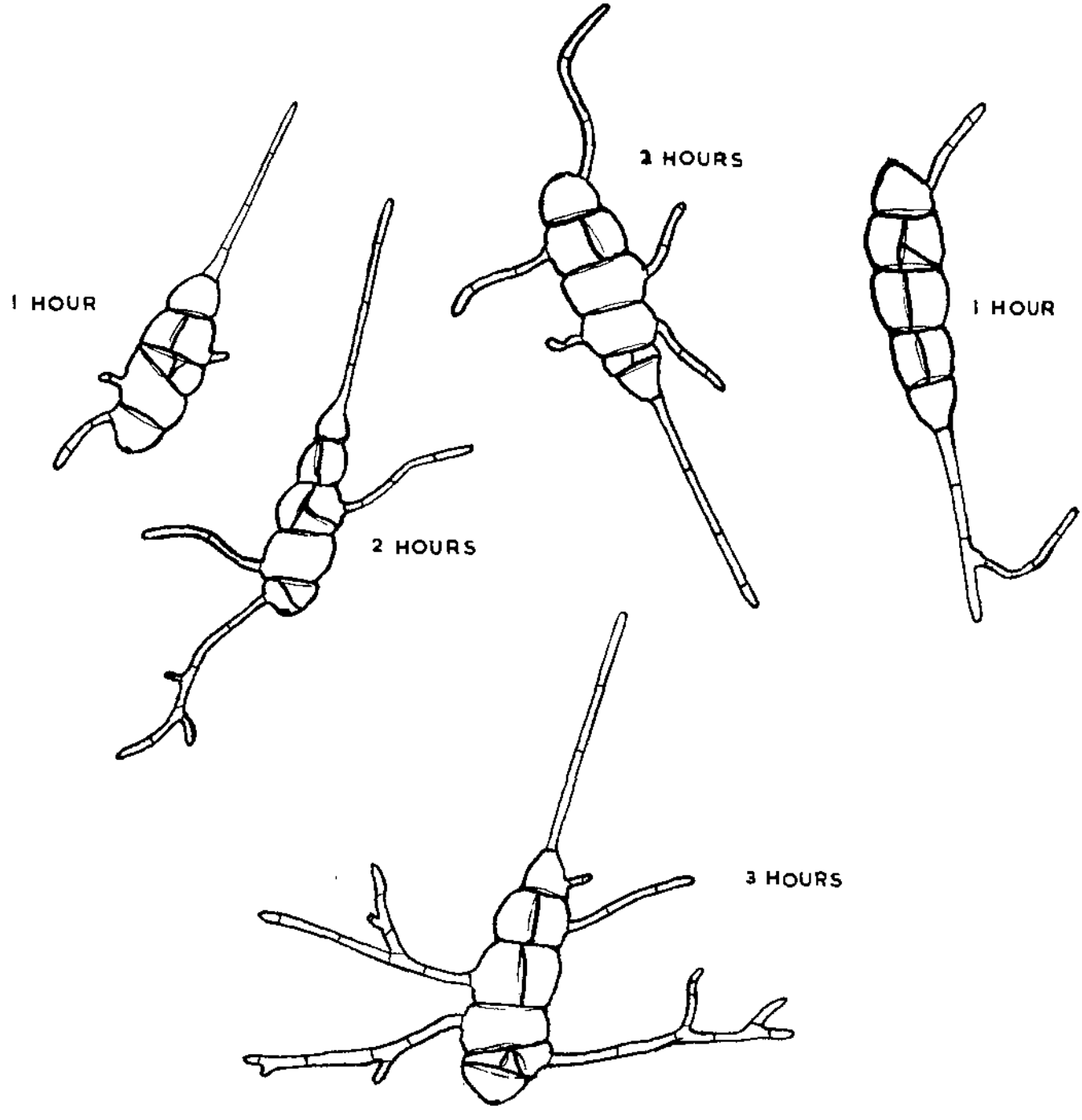
FIGURE 7. GERM TUBE DEVELOPMENT IN SPORE GERMINATION STUDIES.



study some rare instances of germ tube production from the terminal cell of the conidial beak were observed. The methods of germination of spores of A. zinniae are shown in Figure 8.

FIGURE 8

METHODS OF GERMINATION OF SPORES OF A. ZINNIAE ON PDA FILMS AT 28°C.



(b) The effect of low temperature storage on spore germination capacity.

To determine the effect of low temperature storage on spore germination capacity of A. zinniae, spores on films of PDA were stored in petri plates lined with moist filter paper at constant temperatures of -5°C and 1°C. A further set of slides was stored in plates subjected to a 16 hour: 8 hour alternating cycle at temperatures of -5°C and 1°C respectively. Every 24 hours one slide from each of the three series was removed to an incubator at 28°C for 12 hours. Spore germination counts were then made on 100 spores in each of three random low power fields.

Temperatures of storage of -5°C and 1°C inhibited spore germination, but when slides were placed at 28°C for 12 hours many of the spores produced germ tubes. The results of low temperature storage of spores of A. zinniae on PDA films are recorded in Table 14.

TABLE 14. EFFECT OF LOW TEMPERATURE STORAGE ON SPORE GERMINATION.

	Storage Temperature. -5°C	1°C	Alternating cycle -5°C:1°C.
No. days storage before spores produced no germ tubes when placed at 28°C for 12 hours.	26	38	8

Conidia of A. zinniae showed quite long retention of spore germination capacity under constant low temperature conditions. Alternate freezing and thawing of spores severely reduced their longevity and tolerance to low temperatures. This is in accordance with the findings of Cochrane (1960) who noted this effect as a common factor affecting the tolerance of diverse fungi to low temperatures.

Gottleib (1950) states that for many fungi the results of exposure to low temperatures are rarely lethal by their direct action on chemical systems, for they merely retard reactions. Under proper conditions spores can remain viable

for long periods. The lethal effects of very low temperatures can be attributed to the formation of ice crystals in the cell or the membrane as the temperature approaches the freezing point of water.

Spores frozen in water or stored at low temperatures at high moisture content are considered to be more susceptible to low temperature injury than are dry spores (Cochrane 1960). This would indicate that low temperature in itself is not damaging to the spores of many fungi provided that they are stored in a dry condition. This is confirmed in the present study of A. zinniae by the ability of spores to maintain their viability for up to 6 months when stored in a dry condition on cheesecloth at 10°C. - (Page 7).

(c) The effect of relative humidity on spore germination.

The ability of spores of A. zinniae to germinate in atmospheres of known relative humidity was investigated.

Materials and Methods.

Controlled relative humidity conditions within a small closed space were produced with water-glycerine mixtures similar to those used by Wexler & Brombacher (1951). The volumes of each component (in a total volume of 100 ml, at 28°C) used to obtain a required relative humidity (± 1%) were calculated, and are recorded below:-

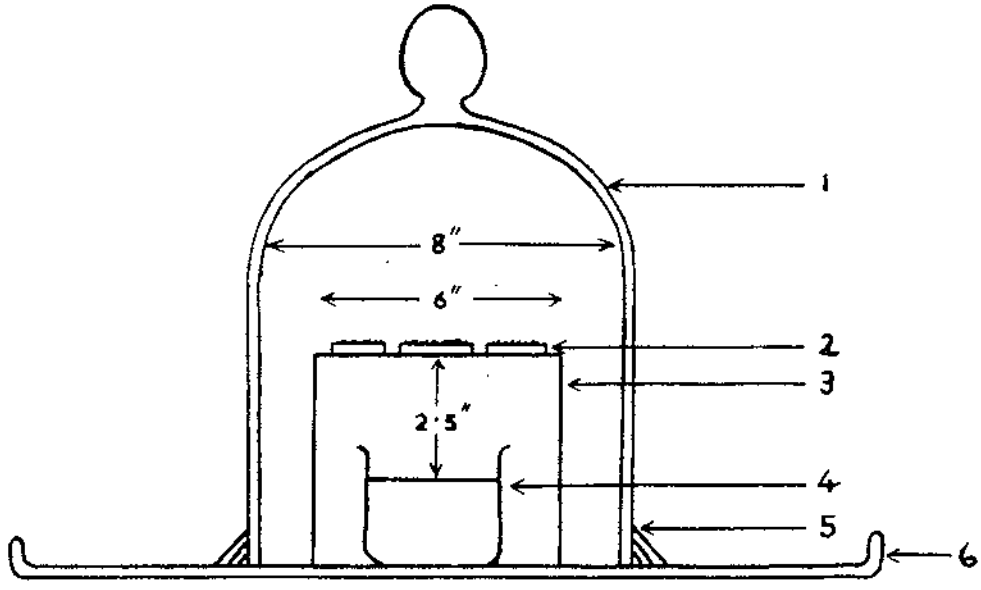
RH%	GLYCERINE (ml)	DISTILLED WATER (ml)
5	95	5
15	92	8
35	84	16
55	72	28
75	55	45
84	42	58
89	32	68
92	25	75
95	15	85
100	0	100

Each volume of 100 ml of glycerine and water was thoroughly mixed in a 250ml beaker. Each beaker was placed under a wire cage (6" x 6" x 5") and dry microscope slides arranged on the top of the cage. The cage was then covered with an 8" diameter glass belljar which was supported on a 12" x 9" plastic tray. The rim of contact between belljar and tray was sealed with petroleum jelly.

The whole apparatus was held at 28°C overnight to allow equilibrium vapour pressure to be obtained within the closed vessel and to ensure temperature equilibration before introduction of spores.

FIGURE 9.

APPARATUS FOR MAINTAINING CONSTANT RELATIVE HUMIDITIES IN SPORE GERMINATION STUDIES



1 GLASS BELLJAR

2 GLASS MICROSCOPE SLIDES WITH DRY SPORES ON SURFACE

3 WIRE MESH CAGE

4 BEAKER CONTAINING WATER-GLYCERINE MIXTURE

5 PETROLEUM JELLY

6 PLASTIC TRAY

Spores were obtained from the surface of 10 day old cultures of A. zinniae which had been scraped, irradiated with ultraviolet light for 1 minute, and incubated in an inverted slanted position at room temperature for 48 hours. These spores were transferred to the surface of the glass slides using a dry camelhair brush. Spores were also germinated on glass slides in distilled water at 28°C as a control.

Spore germination and germ tube development were recorded at 4 hour intervals up to 24 hours, by removing one glass slide from each humidity chamber and counting the percentage of spores germinated in each of three random low power fields.

At first only germination percentage and germ tube length at the end of each incubation period were measured. It was soon realised, however, that the speed of germination and, to a lesser extent, the rate of germ tube growth were more sensitive to relative humidity than were the ultimate germination counts obtained. In observations at 24 hours both of these properties were also measured.

In tests at 100% R.H., fields relatively free from condensation were selected. Germ tube length was measured using a micrometer, 25 germ tubes being chosen at random for measurement.

A conidium was considered to have germinated when the length of any one germ tube exceeded the width of the spore body.

Once a slide was removed from the belljar for measurement of spore germination it was discarded.

Results and Discussion.

This experiment showed that germ tube production, germ tube growth and final germination percentage of spores of A. zinniae were all sensitive to relative humidity. At all humidities up to and including 92% germ tube length was greatly retarded, the number of germ tubes produced per spore restricted and the average percentage germination after 24 hours at 28°C severely reduced.

The results of these studies are recorded in Table 15.

TABLE 15. EFFECT OF RELATIVE HUMIDITY ON SPORE GERMINATION.

Spore germination and development after 24 hours at 28°C:

R.H.%	Average % Germination.	Maximum No. germ tubes per spore.	Max. germ tube length (microns)	Time for 50% spore Germination.
5	0	0	0	-
15	0	0	0	-
35	0	0	0	-
55	0	0	0	-
75	0	0	0	-
84	14	1	90.20	Over 7 days.
89	12	2	88.15	6.2 days
92	22	2	86.10	2.2 "
95	61	6	310.75	20 hours
100	81	7	549.40	16 hours
Control	98	9	Over 600	2 hours

At relative humidities between 84 and 100% the conidia remained turgid and germination proceeded normally. At lower humidities the conidia (especially the conidial beak) and the rudimentary germ tubes shrivelled rapidly. At humidities of 75% or less germ tubes reached a certain length (up to 10 microns) and then stopped elongating and shrivelled. At humidities from 95 to 100% good germination occurred at 28°C, although germination percentage and germ tube growth were less than comparable figures obtained by germinating spores of A. zinniae in distilled water.

It should be emphasised that the results obtained in this experiment are dependent on the criterion, necessarily arbitrary, adopted for considering when spores have "germinated". In the present study the criterion used was that germ tube length should equal or exceed spore body diameter.

If the criterion had been that a spore was considered to have germinated when the sides of the germ tube became parallel, a small number of spores at relative humidities below 84% would have been considered as having germinated, viz. at 5% - 2 spores
15% - 4 spores
35% - 6 spores
55% - 5 spores
75% - 9 spores, compared to no spores at any of these humidities being recorded as having germinated by the criterion actually used in this study.

In the present study it was noted that rudimentary germ tubes produced from spores held at relative humidities of 75% or less for 24 hours were incapable of resuming growth when transferred to drops of distilled water and held at 28°C. Thus these spores were considered not to have "germinated" since they produced onl incipient germ tubes which quickly shrivelled and died. Similar results have beer demonstrated for the germination of conidia of Erysiphe graminis D.C. by Manners & Hossain (1963).

The method of germination of spores in water vapour was similar to that in free water or on agar films, but the time required for the same stage of spore germination to be reached, even at 100% relative humidity, was very much increased. These findings are in general agreement with results reported by Gottlieb (1950).

CONCLUSION.

From this study it appears that the level of atmospheric relative humidity can be a limiting factor to both the rate and extent of germination of conidia of A. zinniae in nature, in the absence of free moisture. Only when the relative humidity is over 95% could this factor be of any practical value to the pathogen through its stimulatory effect on spore germination.

2. PENETRATION AND INFECTION.

The term penetration is used to describe the method by which a pathogen enters host tissue. In this sense it does not include subsequent development within the host (Flentje 1959).

Brown (1936) distinguishes several stages in parasitism, namely the pre-penetration stage, the actual penetration of the host, and the post-penetration stage when the pathogen has gained entry into the host plant.

The pre-penetration stage. Even when the spores have been deposited on a suitable host plant, successful infection is by no means assured. Conditions must be suitable for germination and, as shown in the preceding section, this phase of the Disease Cycle is markedly influenced by environmental conditions.

Penetration involves the mechanism by which a pathogen enters host tissue. Some fungi are "wound parasites", that is, they enter only through a wound or discontinuity in the surface of the host. Many fungi enter the host plant by way of the stomata or through lenticels or water pores (Brown 1936). Others penetrate the epidermis directly through the uninjured cuticle.

Fungi often have characteristic and rather elaborate means of penetration, exhibiting an active response to stinuli (Brown loc. cit.)

Post-penetration. Following penetration, the problem of fungal attack becomes one of actual establishment in host tissue (Eide 1955). This stage of th Disease Cycle has been termed "infection".

Although variously defined by different workers (Gaumann in Eide 1955), Walker 1950), in the present study the term "infection" has been used in the sense prescribed by Walker (1950).

According to Walker (loc. cit.) infection is said to have occurred when the fungus becomes established within the host following penetration and is independent of external food reserves for its continued development.

Because the two processes of penetration and infection are continuous it is often impossible to distinguish the point when penetration has been completed and infection has begun. Accordingly, in the present study, it was not found practical to try to distinguish these two stages because the rate at which both

spore germination and penetration occurred was highly variable within the spore sample used.

Attempts were made to determine the method by which host penetration occurre and also the effect of temperature on the duration and extent of the penetration and infection processes.

(a) Method of Penetration.

To determine the method(s) of penetration involved in the Disease Cycle of A. zinniae a large number of spores were observed germinating on zinnia leaves.

Materials and methods.

Healthy 6 week old zinnia plants were inoculated with a spore suspension containing approximately 6,000 spores per ml. The plants were sprayed with the suspension and held at 75°F throughout the trial. At intervals of one hour a plant was removed from the temperature controlled cabinet and the leaves removed to the laboratory. The surface of the leaves was flooded with acid fuchsin stain and each leaf allowed to drain on a slanted glass slide for 5 minutes. Under the microscope, germ tubes were stained red and easily distinguished from the underlying leaf tissue which remained dark green. The leaf surface was examined under the low power objective of the microscope to locate penetrating spores, which were then examined under higher power.

Observations and Discussion.

In most cases direct penetration was seen to occur, although in some instances penetration by a germ tube entering a stoma was observed. In general direct penetration appeared to be the characteristic method of entry of the pathogen into host tissue.

During germination of conidia several germ tubes developed from different cells of the spore body and spread in an apparently random pattern over the leaf surface.

A number of conidia were seen to be penetrating directly through the

cuticle as soon as 4 hours after spores were placed on the leaf. The tip of the hypha produced an appressorium which was part of the original hypha with an increased diameter.

Direct production of a thin infection peg which penetrated the cuticle and entered an epidermal cell could occasionally be seen. After passing through the cuticle and the outer layer of the epidermal wall the penetration process of the pathogen was considered to be complete.

Stomatal penetration was occasionally observed, hypae forming a globose structure within the stomatal cavity. This structure was considerdd to be either an appressorium or a substomatal vesicle.

Fungal development continued within the cells surrounding the infection site but the mechanism of further development of the pathogen was not clear using the staining method employed.

Direct penetration has also been noted as characteristic of several other Alternaria species including A. solani (Rands 1917), A. cucumerina (Jackson 1959) and A. longipes (Von Ramm 1962).

Direct penetration is reported as being a mechanical process, the appressorium becoming attached to the host surface and providing an adhesive disc against which the infection hypha may push, exerting mechanical pressure on the cuticle during penetration (Wolf & Wolf 1947, Lilly & Barnett 1951, Flentje1959, Dickinson 1960)

Penetration by fungal pathogens through stomata appears to be a more passive process, often influenced by hydrotropic or chemotropic responses. Various stimulus mechanisms have been postulated to explain stomatal penetration --- chemical substances diffusing from the stomata (Bald 1952); water vapour gradients (Dickinson 1949) and stomatal guttation (Bald 1952, Yarwood 1952, Eide 1955).

CONCLUSION.

From this study it is concluded that penetration of A. zinniae into zinnia

leaves is most commonly a direct process, by which the hypha penetrates the cuticle and establishes in the host tissue. In some cases penetration through stomata was observed.

(b) The effect of temperature on penetration and infection.

Studies were carried out to determine the influence of temperature on the rate and degree of penetration and infection by spores of A. zinniae on zinnia leaves. This trial was also designed to determine whether invasion occurred more readily through the upper or lower leaf surface. The method used was similar to that described by Dimock & Osborn (1943).

Materials and Methods.

Six week old zinnia plants, each bearing three pairs of fully expanded leaves, were used in this study. The leaves of the plants were marked for inoculation by drawing (with a 'ball-point' pen) three 5mm circles on the upper leaf surface of one leaf of each pair and three circles on the lower surface of the other leaf of each pair. Thus a total of nine upper and nine lower surface areas were marked on each plant.

One drop of spore suspension was placed with a 80s nozzle dropper on each marked area. The inoculum concentration was such that approximately 10 to 15 spores were present in each drop of the suspension.

Immediately after inoculation each plant was covered with a plastic bag, the mouth of which was tied with string to maintain high humidity. Seven plants were placed in each of six temperature controlled cabinets which were held at temperatures from 60 to 85°F at 5° intervals.

At intervals one plant was taken from each temperature cabinet, the plastic bag removed, and the leaves of the plants dried in front of an electric fan. This prevented further penetration. The plants were then placed on the glass-house bench.

Dimock & Osborn (1943) state that in their studies it was found that,

unless artificially dried, moisture sufficient to permit spore germination, penetration and infection may remain on the leaf surface for a number of hours after it appeafs to the eye to be quite dry.

If penetration and infection had occurred lesions would later develop. These were recorded by direct counts after a period of ten days from inoculation, nine lesions on each of the plant surfaces indicating 100% infection.

Results and Discussion.

Results on this trial are recorded in Table 16.

TABLE 16. THE EFFECT OF TEMPERATURE, LEAF SURFACE INOCULATED AND DURATION OF EXPOSURE ON INFECTION OF ZINNIA LEAVES BY A. ZINNIAE.

Temp. °F ± 2°F	Leaf Surface	Duration of Exposure (hours) 6	8	12	20	24	36	48	Total No. lesions Per surface	Per temp.
60	Upper	0	0	1	4	3	3	5	16	30
	Lower	0	0	1	1	4	5	3	14	
65	Upper	0	1	2	4	2	5	6	20	46
	Lower	0	1	3	3	4	7	8	26	
70	Upper	0	2	6	7	4	8	8	35	74
	Lower	1	3	5	5	7	9	9	39	
75	Upper	1	2	5	6	8	9	9	40	83
	Lower	1	2	4	9	9	9	9	43	
80	Upper	0	1	3	4	3	7	7	25	54
	Lower	0	0	4	5	7	7	6	29	
85	Upper	0	0	2	3	3	4	4	16	34
	Lower	0	0	3	3	3	5	4	18	

Total lesions on upper leaf surfaces - 152

Total lesions on lower leaf surfaces - 169

These results indicate that the optimum temperature for penetration and infection is about 75°F (23.9°C). They also show that at temperatures between 70 and 75°F infection may occur within 6 - 8 hours. Consideration of all temperatures tested suggests that between 60 and 85°F, maximum infection is not reached with a period of treatment of less than 20 hours and usually not until 36 hours after inoculation.

It is interesting to note that the optimum temperature for infection in this study (75°F) is considerably lower than the 82°F which is optimum for the growth of the fungus on culture media (P.D.A.).

In similar studies Dimock & Osborn (1943) reported that the optimum temperature for infection of zinnia leaves by A. zinniae was "probably about 70°F". They also state that invasion occurs more readily through the lower surface of the leaf than through the upper surface. In Dimock & Osborn's experiment there was a 77% increase in the number of lesions developing on the lower surface compared to the number on the upper surface of the leaf. In the present study a much smaller difference (+ 10%) was observed in the number of lesions on the lower surface compared with the upper surface of the leaf.

The stomata on the leaves of Zinnia elegans are more numerous on the lower surface. Since it has already been shown that stomatal penetration does sometimes occur, this increase in the number of lesions on the lower leaf surface in the present study may be due to higher stomatal penetration on the lower leaf surface. However, the low density of stomata on the upper leaf surface would not appear to be a limiting factor to penetration and infection of A. zinniae.

CONCLUSION.

From this experiment it is concluded that the optimum temperature favouring penetration and infection of zinnia leaves by A. zinniae is approximately 75°F. At this temperature the infection period may be as short as 6 - 8 hours.

Infection appeared to occur more readily through the lower surface of the leaves but both leaf surfaces were susceptible to attack.

(c) The effect of leaf moisture on penetration and infection.

An experiment was conducted to determine the length of time plant leaves must be continuously wet for penetration and infection to occur.

Materials and Methods.

Healthy six leaf zinnia plants were sprayed with an aqueous suspension of conidia of A. zinniae containing 12,000 spores per ml. All plants were misted wit a fine spray of sterile distilled water at one hour intervals. Uninoculated plants were also atomised with distilled water as controls. All plants were held in a thermostatically controlled room at 75°F (± 2°F). High humidity was maintained in the room at between 65 and 75% throughout the trial period by placing flat trays of water in front of the fan heater used for temperature control and by standing the pots containing plants in similar trays containing water on a bench nearby.

At one hour intervals three inoculated plants and one control plant were removed from the trays, dried with cold air from an electric fan for 20 minutes and removed to the glasshouse.

Disease rating was recorded after 10 days from inoculation by counting the number of lesions which subsequently developed.

Results and Discussion.

Results of this trial are recorded in Table 17.

TABLE 17. Effect of continuous leaf moisture on the length of the penetration and infection phases of the Disease Cycle.

Time leaves wet. (hours)	Total number lesions on leaves (10 days)
1	0
2	0
3	0
4	0
5	2
6	7
7	19
8	32
9	40
10	47
12	over 50

All controls were disease free.

These results indicate that at the optimum temperature for infection (75°F) at least 5 hours continuous leaf moisture is necessary before the pathogen is capable of infection.

As the period of continuous leaf moisture increased, the number of lesions which subsequently developed also increased. When large numbers of lesions were present on a single leaf it became difficult to identify and count these individually due to coalescence of dead tissue over the leaf surface.

CONCLUSION.

From the results of this trial it is concluded that at the optimum temperature for infection (75°F) leaves of zinnia plants must be continuously wet for a period of at least 5 hours before the fungus can infect host tissue to the stage where it is no longer dependent on leaf surface moisture for its further development.

3. INCUBATION.

The incubation period is defined as the interval between infection and the appearance of symptoms of the disease (Walker 1950).

During the period of incubation the pathogen proliferates and spreads in the host tissue. These processes consist of biochemical and physiological reactions inside the tissues and cell protoplasts. They are often inconspicuous and difficult to follow (Kern 1956). Only at the end of the incubation phase of the Disease Cycle do morphological alterations become visible on the plant.

The visible symptoms of the disease are often unspecific and cannot give much information on the mechanisms of pathogen action. External symptoms on the plant are the expression of death of cells or tissues and the specific reactions may already be over.

For many diseases the sequence of visible events is well known, but about the reasons and factors which determine this sequence of events, only scattered information is available.

For the invading pathogen, the host plant represents a nutrient substrate to feed on. The presence of suitable nutrients and the availability of the parasite to utilise these under the given conditions are an important pre-requisite for its growth and proliferation (Kern 1956).

During the incubation period the host plant may answer the pathogen's action with defence reactions. In many cases, however, protoplasts become more and more damaged, morphological alterations of cells and tissues become visible and the period of manifest disease begins.

(a) Pathogen development during the incubation period in host tissue.

Following penetration of either the upper or lower leaf surface of zinnia plants the pathogen appeared to progress some distance from the infection site prior to the appearance of manifest symptoms. To try to determine the sequence of events during the incubation phase of the Disease Cycle a whole leaf clearing method similar to that described by White & Baker (1954) and Shipton & Brown (1962) was used.

Materials and Methods.

Leaves of healthy zinnia plants were inoculated by spraying with a spore suspension of A. zinniae containing 12,000 spores per ml. At one hour intervals inoculated leaves were removed from a plant, cut into approximately ½ inch strips and immersed in 10 - 15 ml. of alcoholic lactophenol cotton blue. (Lactophenol cotton blue; 95% alcohol 1:2). The lactophenol cotton blue was prepared as reported by Shipton & Brown (1962);

Phenol	10 gm.
Glycerine	10 ml.
Lactic acid	10 ml.
Aniline blue	0.02 gm.
Distilled water	10 ml.

The solution, containing the leaf pieces, was brought to boiling point and simmered for 1 minute. Leaves were allowed to remain in the stain for 1-2 hours at room temperature. They were then removed, rinsed in water and placed in chloral hydrate (5gm chloral hydrate + 2ml distilled water) for 30 minutes. The pieces were then mounted on microscope slides in glycerine.

The occasional failure of the chloral hydrate to clear cells was remedied by repeating the boiling in alcoholic lactophenol cotton blue and clearing again in chloral hydrate.

Using this method it was possible to gain some information on the method of development of the pathogen in host tissue.

Observations and Discussion.

After penetration profuse mycelial growth permeated the leaf tissue, radiating inter- and intra-cellularly from the point of entry with little or no apparent limitation by the cell walls. Ultimately there was a progressive collapse of epidermal cells, beginning from the infection site.

After the host cells collapsed the pathogen grew quickly in dead tissue. The epidermal cells seemed to be particularly susceptible and were often attacked first. This was followed by the collapse of pallisade cells, which in some cases retained their integrity to some extent.

The mycelium of the pathogen appeared inter- and intra-cellularly in

cleared leaf tissue as light brown threads ramifying throughout the epidermal and mesophyll regions of the host.

As soon as cell death commenced it became more difficult to observe the mycelium of the pathogen due to the masking effect of dead protoplasts.

(b) The effect of temperature on the duration of the incubation period.

Incubation is generally a rather extended process and considerable variation in its duration is frequent. With the pathogen established in the host tissue temperature, rather than humidity, becomes the most important factor affecting the length of the incubation period. (Waggoner 1960).

Observations were made of the effect of temperature during the incubation period on the minimum time before inoculated plants developed macroscopic symptoms.

Materials and Methods.

Healthy zinnia plants at the six leaf stage of development were inoculated with a conidial suspension of 12,000 spores per ml. Plants were held at controlled temperatures of 50, 60, 70, 75, 80 and 85°F throughout the trial period. Frequent observations of inoculated plants at each temperature were made. By marking lesions with a 'ball-point' pen as soon as they appeared on the leaves it was possible to determine when further lesions developed. Plants sprayed with sterile distilled water were also held at each temperature as controls.

The number of hours after inoculation when the first macroscopic lesions became visible was recorded.

Results and Discussion.

The results of this trial are recorded in Table 18.

TABLE 18. (See next page.)

TABLE 18. Length of Exposure of Inoculated Plants at Different Temperatures before Macroscopic Lesions become Visible on Leaf Tissue.

Temperature °F.	Hours Exposure Before First Lesions Visible.
50	approx. 120
60	73
70	48
75	50
80	74
85	106

From these results it was possible to calculate the approximate length of the incubation phase of the disease cycle. In the previous section on penetration and infection it was shown that these two processes were completed after the following periods, dependent on temperature:

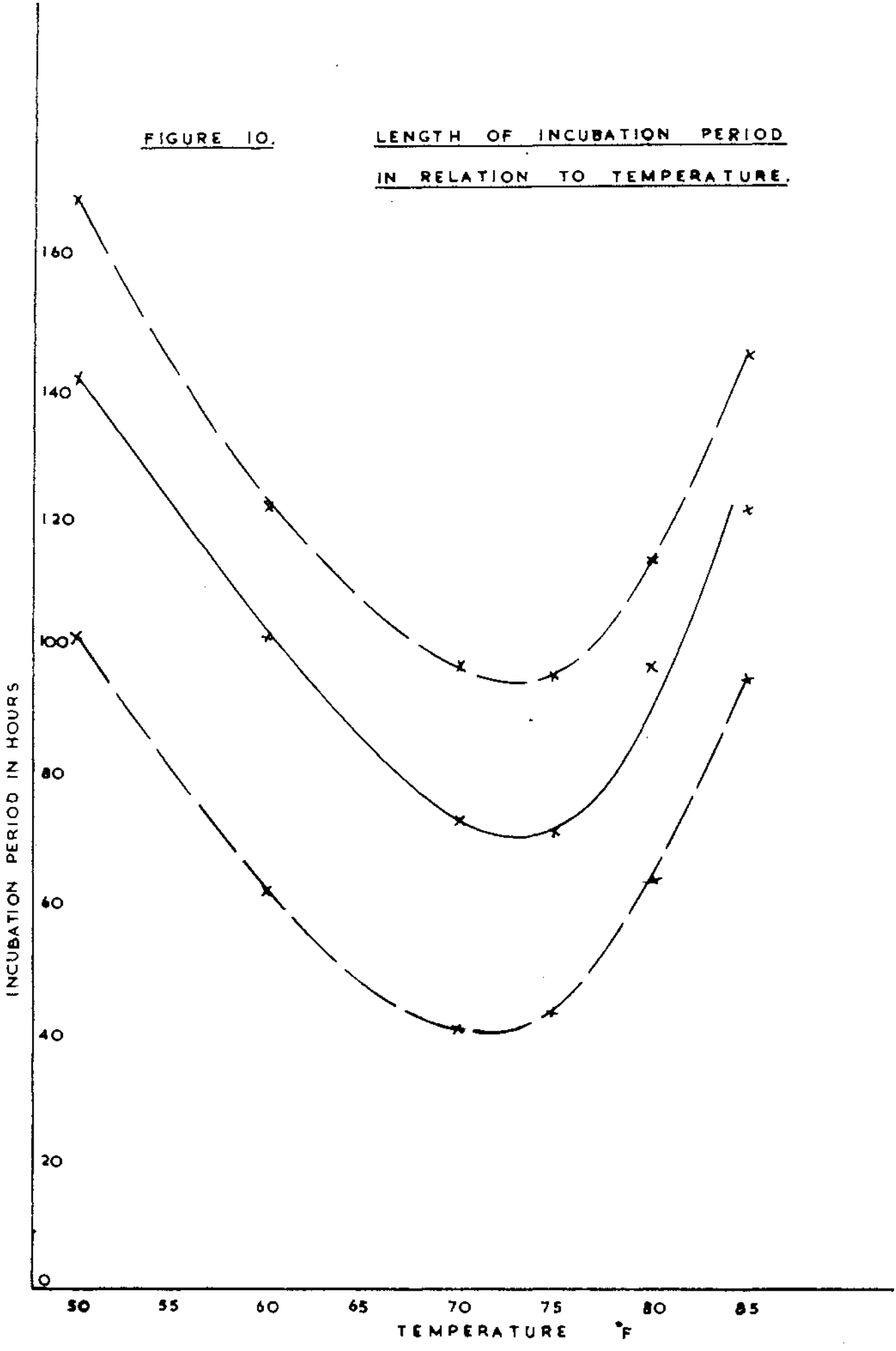
50° F	20 hours
60° F	12 "
70° F	6 - 8 hours
75° F	6 - 8 "
80° F	8 - 11 "
85° F	12 hours

By subtracting these times from the shortest period required for disease manifestation at different temperatures the minimum length of the incubation phase of the disease cycle could be calculated. The length of the incubation period in relation to temperature is shown in Figure 10. The broken lines represent the boundaries of observed minimum and maximum length of the incubation period and the solid line the approximate period at which maximum expression of lesions was observed at various temperatures.

At the range of optimum temperature for infection (70 - 75°F) the minimum length of the incubation period appeared to be approximately 40 hours.

At 70° F complete expression of symptoms occurred about 24 hours earlier than at 60° F, while the incubation period lasted approximately 3 days when

FIGURE 10. LENGTH OF INCUBATION PERIOD IN RELATION TO TEMPERATURE.



plants were held continuously at 50°F.

During this trial it was noted that lesions produced on the younger, softer leaves near the growing apex were invariably larger and visible sooner than lesions on the more mature leaves of inoculated plants. This may be attributed to the greater ease and earliness of penetration of germ tubes of the pathogen through the thinner cuticle of younger leaves than of older leaves. Conversely the effect may be due to a reduction in distribution or amount of nutrients in older leaves favouring the growth of the pathogen in host tissue.

As the length of time from inoculation increased there was a progressive increase in both the size and number of lesions produced, over the temperature range studied. At the optimum temperatures for disease development (70 to 75°C) all plants showed complete leaf collapse within 10 days. At temperatures above 80°F the size of lesions was greatly reduced.

CONCLUSIONS.

Although the growth and spread of A. zinniae in zinnia leaves is difficult to follow, it is concluded that the pathogen progresses some distance from the infection site before macroscopic symptoms appear. Mycelium of the pathogen is both inter- and intra-cellular in host tissue, epidermal cells being especially prone to early collapse.

At the range of optimum temperature for infection (70 to 75°F) the minimum length of the incubation period is approximately 40 hours and the length of time before symptoms appeared increased at temperatures higher or lower than the optimum.

As the length of time from inoculation increases there is a progressive incfease in the size and number of lesions produced.

This study shows that the incubation phase is the most extended single step in the development of the disease cycle. The length of incubation is greatly dependent on temperature and the size and number of lesions which subsequently develop on leaves following inoculation is closely related to both temperature and time.

4. MANIFESTATION.

The term manifestation refers to the expression of physiological disturbance and is applied to evidence of the presence of a disease inducing agent (Chester 1959).

Manifestation of disease on zinnia plants caused by A. zinniae involves spotting of leaf, stem and blossom tissues. The precise nature of this expression on different host organs has been described in detail in Part A of this chapter (page 61).

5. PRODUCTION OF INOCULUM.

Consideration was given to the method of production of conidia of A. zinniae. Conidia may be produced during the period of manifestation or at a later stage, and are formed singly at the tips of conidiophores arising from dead host tissue.

Microscopic examination of cleared infected host tissue revealed the presence of light brown inter- and intra-cellular hyphae within the host and brown septate conidiophores arising from the stomata. Spores of the fungus were often visible c fresh specimens of infected leaves when atmospheric conditions favoured sporulation.

The most active region for conidial production was at the site of original infection and the density of spore production on other areas of a lesion was extremely variable.

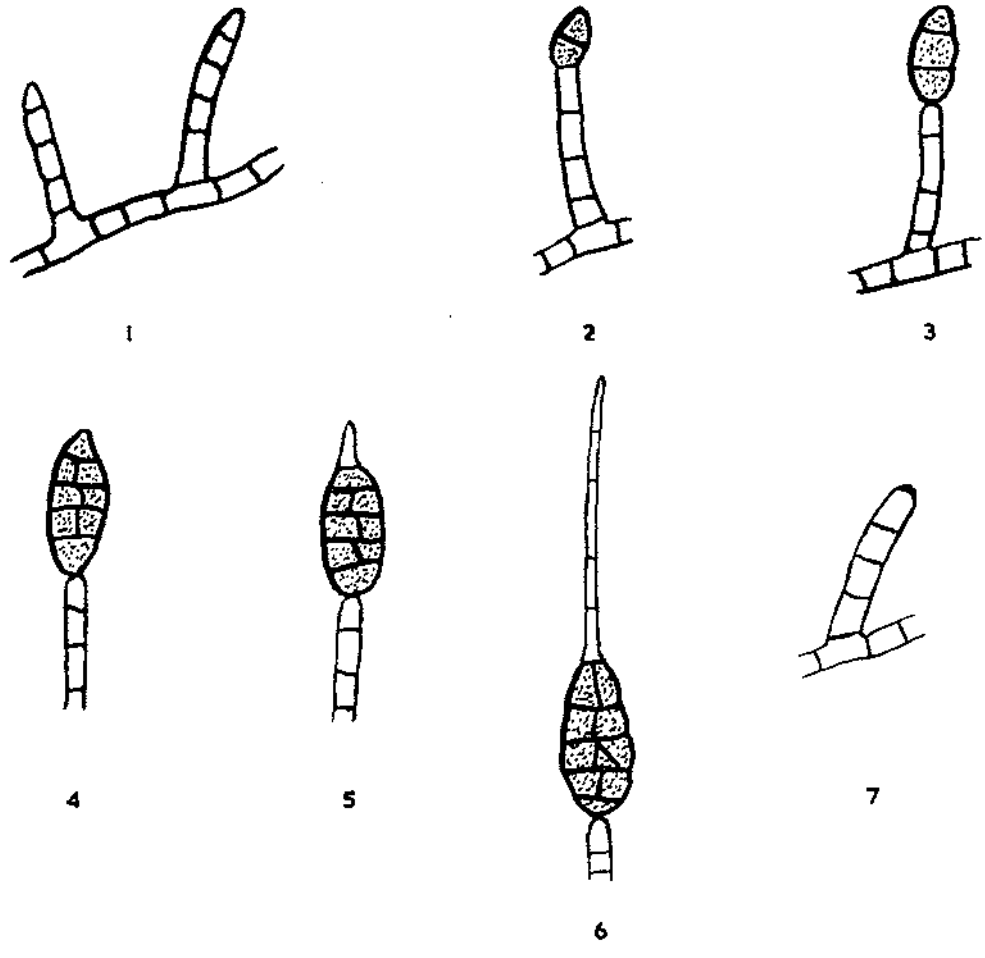
When plants were held continuously under high humidity conditions at favourable temperatures of 70 to 80°F production of spores was seen to occur within two days of the first appearance of lesions, or approximately 4 to 5 days from inoculation.

Normal humidity conditions encountered in the glasshouse were not high enough to support conidial production. If diseased plants were subjected to high humidity, either by covering while wet with plastic bag or by use of a humidity cabinet, sporulation on lesions occurred within 36 hours, at normal glasshouse temperatures of 50 to 80°F.

During the summer months, when it was necessary to damp down the glass-house to reduce the temperature, some secondary spread of A. zinniae was noted in zinnia plants stored on the glasshouse benches. This would indicate that the pathogen was capable of sporulation, dissemination and of infecting healthy host tissue under such conditions. Thus it seems probable that high humidity is a pre-requisite for the production of conidia from lesioned host tissue.

The sequence in the development and production of conidia of A. zinniae is presented in Figure 11.

FIGURE II. SEQUENCE OF CONIDIAL DEVELOPMENT.



CAMERA LUCIDA DRAWING OF CONIDIA IN DIFFERENT STAGES OF DEVELOPMENT.

1-6. PROGRESSIVE FORMATION OF A SINGLE CONIDIUM FROM THE APICAL CELL OF A CONIDIOPHORE.

7. CONIDIOPHORE FOLLOWING CONIDIAL ABSCISSION SHOWING TERMINAL SCAR.

6. EXIT FROM THE HOST AND DISPERSAL OF INOCULUM.

When the host plant becomes diseased the causal organism frequently produces a crop of spores which function as the secondary inoculum and serve to initiate secondary infections. Secondary cycles may be repeated several times during the growing season and are the means whereby there is rapid and widespread buildup of a particular disease during the relatively short period that host plants are available.

In general, the method of dissemination of secondary inoculum will be important in determining the extent and range of inoculum dispersal. The spread of spores of a pathogen which is dependent on water splash effects, for instance, will tend to be strictly localised. Windborne spores, on the other hand, may be carried long distances and hence secondary cycles may produce inoculum which is capable of causing infection to plants some distance from the original site of inoculum production.

The various agents available for the dispersal of conidia of A. zinniae include wind, rain, insects, man and cultural practices. Field observations on the pattern of spread of Alternaris leafspot had lead to the conclusion that the last three of these agencies were unimportant in the dissemination of the pathogen. Thus wind and rain were considered in this study as the two major factors which could be important in conidial dispersal. The study was conducted in two sections:

(a) Laboratory trial
(b) Field trial.

(a) Laboratory Trial.

Observations were made on the effect of wind and water on the liberation and spread of conidia of A. zinniae.

Materials and Methods.

Zinnia leaves from infected plants were held at 28°C under high humidity conditions for 48 hours. These leaves were then examined under a binocular microscope and sporulation was shown to be heavy. Ten of those leaves which were

showing heavy spore production from disease lesions were placed on an 8 x 8.5cm glass slide and secured in position with adhesive tape. The slide was held in a clamp, 4 inches from a 'Kosyaire'+ fan. Pots, each containing 3 healthy zinnia plants, were placed 9 inches from the glass slide and 13 inches from the fan so that plants and slide were in the direct path of the air stream.

The trial was carried out in six sections, the exposure time in all cases being 10 minutes.

1. Dry air from the fan was passed over the sporulating leaves on the glass slide. The leaves of the test plants were dry.
2. Dry air from the fan was passed over the sporulating leaves, the leaves of the test plants being wet during the test period.
3. A fine mist spray of distilled water was directed into the air stream from the fan between the fan and the glass slide bearing sporulating leaves. The leaves of the test plants were dry.
4. As for 3 except that the leaves of the test plants were misted with distilled water prior to exposure.
5. Water was directed onto the sporulating leaves and allowed to splash directly onto the test plants. No air movement from the fan was used. The leaves of the test plants were dry prior to treatment.
6. As for 5 except that the leaves of the test plants were misted with distilled water prior to treatment.

Following exposure all plants were held in a moist chamber under high humidity conditions for 36 hours at 70°F and then removed to the glasshouse bench. Disease counts, as shown by the number of leaves which developed lesions, were made 10 days after exposure. Reisolations of the pathogen were also carried out.

+ 'Kosyaire' is the trade name of a domestic electric fan manufactured in New Zealand.

Results.

The results of this trial on the method of spore dissemination of A. zinniae are recorded in Table 19.

TABLE 19. Lesion Development on Plants Subjected to Different Secondary Spread Treatments, 10 days from Treatment.

Treatment.	No. Plants Exposed.	Total No. Leaves.	No. Leaves Infected.	% Infection	Disease Rating.
1	12	92	0	0	0
2	12	100	0	0	0
3	6	56	22	39.3	4
4	6	68	22	32.4	4
5	12	108	97	89.8	9
6	6	68	62	91.1	10

Disease rating was calculated on a scale 0-10; 0 indicating nil infection and 10.90 - 100% infection, one lesion per leaf being sufficient to record that particular leaf as being diseased.

These results indicate that air movement alone (treatments 1 and 2) is of no importance in the dissemination of spores of A. zinniae. Whether this is because wind force is insufficient to dislodge spores from their conidiophores or because the conidia are too heavy or aerodynamically unsuited to airborne dispersal was not ascertained.

It appears that the most important mechanism of spread of conidia is by direct splash effects of rain or watering of plants. Wind blown water drops containing spores may also be of some importance. Water appears to be important not only in dissemination but, as indicated by observations in this trial, also aids in the mechanical removal of conidia from the conidiophores.

Direct splashing of water from sporulating leaves to nearby healthy leaves, with or without the aid of wind, appears to be the most important mechanism of conidial dispersal.

In the present trial no great variation was found between the amount of infection occurring on plants which were dry during the exposure period and those which were atomised with distilled water prior to exposure.

(b) Field Trial.

Water drops running off the leaves of plants on which there were sporulating lesions were caught in a test tube and the runoff water examined under a binocular microscope. Spores were found to be present in large numbers. This observation would indicate that water movement is important in the spread of A. zinniae and prompted the setting up of a field trial to try to relate field weather conditions of rainfall and wind speed with spore dispersal.

Attempts were made to correlate numbers of conidia found on glass slides smeared with Vaseline placed within a crop of diseased zinnia plants, with prevailing weather conditions.

Materials and Methods.

The experimental area had not been used for growing zinnias for at least 15 years and possibly never before. The area was in a sunny location with fertile soil. The plants were transplanted into this area when 4 weeks old (four leaf stage) into 4 rows of 30 plants, with 12" between plants and rows.

Glass microscope slides 75 x 25mm were held with a spring clip, the top of the latter being secured to a bamboo stake with a drawing pin. A rubber band around the top of the clip held the clip and slide in a vertical position. The bamboo stake was placed so that the bottom of the slide was 6 inches above ground level.

PLATE 14. Vaselined slide in position in a diseased zinnia crop, showing apparatus used in field trial.

Six slides were placed in the crop at approximately 4 ft. intervals. At 48 hour intervals the slide from each clip was removed to the laboratory and a further series of clean slides inserted in the clips in their place. Slides were examined microscopically and counts of the numbers of conidia present on each slide were made. The total number of spores on all six slides was expressed as an average.

The trial was run for 46 days, during which time efforts were made to relate the average number of spores on the slides with weather records obtained from Grasslands Division, D.S.I.R., approximately one mile from the site of the experiment.

Results.

In many cases spores were found on slides in clumps containing as many as 20 or 30 spores. This would indicate that water containing conidia had been deposited on the slide and the water had subsequently evaporated.

Results of this field trial are presented graphically in Figure 12.

Figure 12 substantiates the findings of the previously described laboratory trial on the method of dissemination of conidia of A. zinniae. Of the various agencies which may cause conidial dispersal, water, in the form of rain drops may spread conidia over the plant and to nearby plants; air movement does not appear to account for the dispersal of spores.

In all test periods when rain fell, there was a sharp increase in the number of conidia observed on the slides placed in the crop. When no rain fell during the test period and even when wind speed was as high as 15 m.p.h. (e.g. test period 17), the number of conidia on the slides was low. The occurrence of some spores on slides following test periods with no rain but high wind speeds may have been due not to wind dissemination of conidia but rather to leaves bearing spores being blown against the slides. This assumption was based on the fact that during test periods of heavy wind speeds isolated smears in the vaseline, with spores adhering, were observed on the slides. Thus the contact of infected leaves bearing spores with nearby healthy leaves during periods of high winds cannot be excluded as a possibility for the spread of the pathogen in nature. This is especially probable in closely planted beds.

In most cases, as the amount of rain which fell during a test period increased there was a corresponding increase in the number of spores found on the slides, irrespective of wind velocity.

Discussion.

Some confusion exists in the literature as to the method of dissemination of spores of A. zinniae throughout the growing season. Baker (1956) for instance, states that isolation between crops is necessary to control A. zinniae "because of the hazard of spores being blown to clean stock from infected crops".

FIGURE 12 RELATION OF SPORE NUMBERS ON VASELINED SLIDES TO BOTH RAINFALL AND WIND SPEED

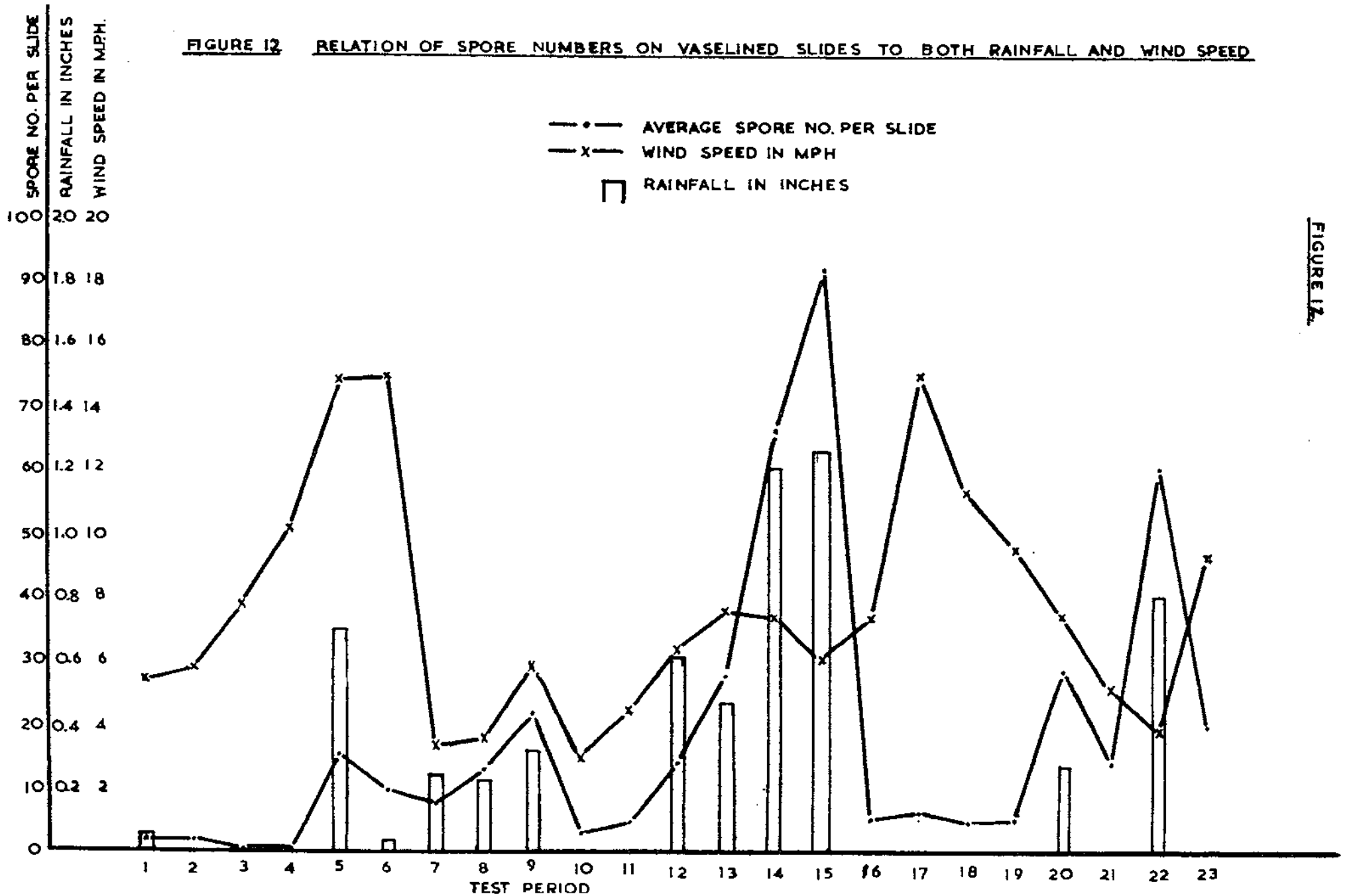


FIGURE 12.

Field observations, as well as the two trials outlined above, indicate that water splash dissemination of conidia of the pathogen is most likely and that wind, apart from the blowing of water drops containing spores over short distances, is of little importance.

Rarely is stem lesioning observed on seedlings which do not manifest cotyledonary lesioning. This would indicate that spores may be carried down the stem in water droplets from the infected cotyledons above.

During this study it was noticed that some plants possess a slight groove running longitudinally down the length of the stem. This stem grooving did not appear to be a consistent characteristic of any one variety of Zinnia elegans, but occurred in isolated plants within different varieties. These grooves were shown to act as an effective water race down the stem. Adult plants which possessed these natural stem grooves were stripped of their leaves and a spore suspension (20,000 spores/ml.) was poured onto the stem apex using a fine nozzled dropper and allowed to run down the grooves. The plants were held under high humidity conditions for 48 hours and then placed on the glasshouse bench. All plants developed heavy stem lesions around the grooves, illustrating that spores carried down the stem in water droplets can cause stem lesioning.

From observations on the growth of infected seed in sterilised soil in the glasshouse it was seen that when very high seeding rates were sown to produce a dense stand of seedlings, a high proportion developed stem lesions, mainly at or just above ground level. Some seedlings, however, also showed stem decay some distance from ground level. Under such conditions stem lesioning may be observed on some seedlings which do not show any cotyledonary decay. Such lesioning was considered to be caused by water drops containing spores running off diseased cotyledons onto the stems of plants below, during overhead watering.

Under the lower seeding rates normally used, however, stem lesioning was rarely observed on plants not showing cotyledonary infection.

In this study it was also shown that on a seedcoat from a diseased seed,

which had fallen to the ground from a lesioned cotyledon, the pathogen may sporulate under the high humidity conditions near the soil surface. Under conditions of overhead watering widely used in nurseries spores may be splashed onto the stems of developing seedlings.

Although no work was carried out to ascertain the importance of this method, it is also possible that spores falling or being washed from infected leaves to the soil surface may subsequently be splashed up onto the stem in association with soil particles. Alternatively, spores on the soil surfact may germinate and the resultant mycelium may invade stem tissues at ground level.

CONCLUSIONS.

Since conidia of A. zinniae are apparently not wind disseminated, the secondary spread of spores by rain splash effects may be considered to be strictly localised. Long distance spread of the pathogen to new areas could thus only occur through the transportation of diseased plants or soil containing spores, or by the use of seed harvested from diseased plants.

From observations on the spread of the disease in glasshouse sowings it is concluded that several mechanisms are available for the spread of the pathogen:-

1. Splashing of spores from infected leaves to the leaves or stems of plants.

2. Washing of spores from infected leaves down the stem of the infected plant.

3. Water splashing of spores produced on diseased testa tissues which have been shed to the ground.

4. Possibly through spores being washed to the soil surface and either being splashed onto the stems of plants during watering, or infecting the stems by means of mycelium arising from spores which may have germinated on the soil surface.

7. METHOD BY WHICH THE PATHOGEN BECOMES SEEDBORNE.

Seedborne transmission of plant pathogens is extremely important because, (i) the host and parasite are closely associated, providing maximum opportunity for infection, (ii) spread over long distances is possible, and (iii) the fungus strain best adapted to a given variety stays with it, increasing the chance of an epidemic (Baker 1952).

The fact that blossom lesioning with A. zinniae invariably affects the prominent ray florets to which the seed is attached, would indicate that the seed can become diseased during the period of seed development.

A. zinniae is associated with the seed of Zinnia elegans in two ways (page175

1. As surface borne spores adhering to the seedcoat.
2. As internally borne vegetative mycelium established within the tissues of the seed.

The zinnia belongs to the Compositae and has its flowers arranged in a close head composed of pistillate ray flowers and disc flowers containing both pistil and stamens.

From observations in the field, the mechanism by which the pathogen becomes seedborne is considered to be as follows ---

The petals of the ray florets, being slightly curved, act as moisture traps, conveying spores produced on lesioned florets down to the developing seed, and providing the moisture necessary for spore germination and penetration of the seed which is in the early stages of development. It has been observed that moisture may remain in the central portion of the flowerhead for long periods after the leaves and florets of zinnia plants are apparently dry following rain. This feature is especially pronounced when heavy dew or misty rain occurs during the flowering period. Thus wet weather during flowering and seedset may play an important part in determining the amount of seed which is subsequently diseased.

The seed is closely associated with the florets, being separated only by a short stalk. This latter is often infected by the pathogen and shows typical disease lesioning.

The fact that the junction between petal and seed is ruptured during threshing, leaving a ragged end to the majority of seeds, may provide a convenient area for the trapping of external spores on the outside of the seed coat during the harvesting process.

To obtain some information on the method of seed carriage of A. zinniae two aspects of study were carried out.

1. The effect of flower age on susceptibility to floral attack.
2. The effect of stage of seed development on susceptibility to attack and on the site of the pathogen associated with the seed.

1. The effect of Flower Age on Susceptibility to Floral Attack by A. zinniae.

On zinnia blossoms the first sign of disease visible to the naked eye is the appearance of minute dark lesions. Under favourable environmental conditions these can appear 48 hours after inoculation. They subsequently enlarge and coalesce to form spreading brown to black lesions. Ultimately the individual florets collapse, making the blossom unsightly. In wet weather the diseased flowerheads become watersoaked and provide an ideal substrate for such other fungi as Alternaria tenuis and Botyrtis cinerea.

Materials and Methods.

Approximately 85 plants were used in this study. The plants were allowed to develop in their natural form, no attempt being made to increase the number of flowers produced on each plant by "pinching back". This allowed the production of one terminal flowerhead per plant. The date of flower-bud burst was recorded for each flower. This date was recorded as soon as the bud scales had separated to expose the inflorescence. A wide range of varieties and bloom colours were included in the trial, allowing a progressive increase in the age of flowers as determined by the number of days from bud burst.

All plantswere inoculated with a spore suspension of A. zinniae, containing 45,000 spores per ml., and held in a polythene humidity cabinet for 4 days. The plants were then removed to the glasshouse bench. A disease rating was obtained 10 days after inoculation by counting the number of infected ray florets and

expressing these as a percentage of the total number of ray florets in the head.

RESULTS and DISCUSSION.

The results of incidence of floral attack by A. zinniae 10 days after inoculation are presented in Table 20.

TABLE 20. INFECTION OF ARTIFICIALLY INOCULATED ZINNIA BLOSSOMS OF DIFFERENT AGES.

Age in days.	No. flowers Inoculated.	Total no. Florets.	No. infected Florets 10 days.	% Infection.	Stage of Development.
Unopen	2	91	2	2.2	Green bud.
1	1	30	3	10.0	Coloured bud.
4	1	54	4	7.4	
5	1	73	16	22.2	
8	2	47	11	23.4	Half open bloom.
10	1	48	5	10.4	
12	4	216	41	19.0	Mature bloom.
14	2	92	12	13.0	
15	4	150	20	13.3	
16	2	158	27	17.1	
19	6	370	103	27.9	
20	2	180	37	20.6	
22	6	388	121	31.2	
25	6	427	198	43.6	
26	2	100	56	56.0	
29	5	264	153	57.9	
31	2	75	32	42.7	
33	8	255	170	66.7	
35	2	78	63	80.8	
36	7	380	241	63.4	
39	4	129	101	78.3	

(Continued overleaf)

TABLE 20 (Continued).

Age in Days.	No. flowers Inoculated.	Total No. Florets.	No. Infected Florets 10 days.	% Infection.	Stage of Development.
40	4	299	245	81.9	
41	3	178	164	92.1	
42	3	133	119	89.5	
44	3	183	165	90.2	
46	1	46	42	91,3	

These results indicate that there is a marked increase in susceptibility of flowers with age. Whether this is due to a change in the chemical composition of florets with age or to a senescence effect was not determined.

It appears that flowerheads of Zinnia elegans show an age effect in their susceptibility to the disease and that this must be a factor to take into consideration in studying seed attack by A. zinniae.

2. Stage of Seed Development - its Effect on Disease Incidence and the Site of Seed Attack by A. zinniae.

Materials and Methods.

The blossoms of healthy zinnia plants were sprayed with a spore suspension of 10,000 spores per ml. Seed was hand collected from these inoculated flowers after 14 days and separated into four categories according to stage of development

Group.	Seed Colour.	Endosperm development.	General Classification.
1	white	Nil	Undeveloped.
2	green	some	'milky' stage.
3	brown	Seed fully swollen, but soft.	Immature.
4	Dark brown.	Complete, seed hard and dry.	Mature.

Seed from each group was studied to determine the incidence of A. zinniae associated with the seed. Half of the seed in each group was plated directly to prune agar. The other half was dusted with Fernasan + to kill any surface borne conidia of the pathogen and then plated to prune agar.

All plates were incubated at 28°C for 5 days and the number of colonies of the pathogen developing from the seed was recorded.

Control samples of the seed were obtained by spraying healthy flowerheads with distilled water, collecting seed after 14 days and dividing it into the above four groups. 'Control' seed in each group was tested for A. zinniae by the same methods as for seed obtained from inoculated flowerheads.

Results and Discussion.

Results of growth of colonies from seed are presented in Table 21.

TABLE 21. EFFECT OF FLOWERHEAD AGE ON SEED INFECTION.

Group.	Seed from Inoculated Flowerheads.		Seed from 'control' Flowerheads.	
	No. seeds plated directly.	No. seeds dusted and plated.	No. seeds plated Directly.	No. seeds dusted and Plated.
1	39	40	40	40
2	117	117	40	40
3	77	77	40	40
4	36	50	40	40
	Percentage A. zinniae from seed (5 days)		Percentage A. zinniae from seed (5 days)	
1	18.6	12.0	0	0
2	23.9	9.8	0	0
3	28.6	7.8	2.5	0
4	52.8	2.0	2.5	0

+ Fernasan - An I.C.I. product containing as active ingredient Tetramethyl thiuram disulphide 50%.

These results indicate that with seed plated directly to prune agar, the percentage of A.zinniae as determined by the number of colonies of the fungus arising from the seed is increasingly higher in inoculated seed with increasing seed maturity. This high recovery of the pathogen is considered to be due to the presence of both internally and externally borne inoculum.

With inoculated seed which had been dusted to kill all surface borne inoculum the percentage of A. zinniae associated with the seed was shown to decrease with increase in seed maturity. No detection of A. zinniae was recorded in control samples of seed which had been dusted, and only a small percentage of the pathogen was recovered from 'control' seed plated directly to agar.

These results show that as seed matures the percentage of internal infection of seed decreases and the total disease incidence increases due to the presence of loose spores adhering to the seedcoat surface. Some seed infection was shown to occur even in mature seed. This indicates that, although the stage of seed development markedly influences the relative proportions of surface and internally carried seedborne inoculum, the mechanical barrier presented by the seedcoat at maturity is insufficient to completely prevent the pathogen penetrating and establishing in seed tissue.

Thus it is concluded that as seed matures in the flowerhead its susceptibility to infection by A. zinniae is greatly decreased, but the amount of the pathogen associated with the seed as surface borne spores may be very high. Thus it appears that in order to control seed attack by A. zinniae it is essential to prevent seed infection in the early stages of maturity and to control or prevent seed contamination immediately prior to and during seed harvest.

8. SURVIVAL OF ALTERNARIA ZINNIAE IN THE ABSENCE OF ZINNIA PLANTS.

Fundamental knowledge of the methods of survival of micro-organisms in the absence of the host crop is often the key to eventual development of plant disease control measures. Many methods are available for the survival of fungal pathogens from one season to another - in the soil, on plant debris, on alternate hosts or by means of carriage with the seed.

Since A. zinniae is seedborne, experiments were conducted to determine the capacity for survival of spores present as loose conidia adhering to the seed surface and of mycelium borne internally in zinnia seed.

Because of the manner of disease development observed in zinnia crops grown in the same area in successive seasons, the soil was also suspected as being one of the sources of primary infection. Investigations were therefore carried out to determine the survival ability of conidia of A. zinniae under laboratory conditions, whether asexual spores could survive between crops in the soil, and the ability of A. zinniae to survive as vegetative mycelium in the soil or in crop debris.

(a) SURVIVAL AS INOCULUM ASSOCIATED WITH ZINNIA SEED.

Neergaard (1945) and Baker & Davis (1950) have reported that A. zinniae can survive with the seed of Zinnia elegans for about 5 years. Accordingly studies were made to determine the survival of loose spores adhering to the seed surface and of internal mycelium of A. zinniae present within the seed.

(i) Survival as Surface Borne Conidia.

The technique used to determine the length of time loose spores of A. zinniae could remain viable on seed was essentially the same as that used by Bolley (Cole and Couch 1958) for determining the presence of spores of the wheatbunt organism (Tilletia foetida Wallr) and the flax wilt fungus (Fusarium oxysporum f. lini Bolley) on seed of their respective hosts. This method consisted of washing seed with distilled water and centrifuging the wash water to accelerate the

sedimentation of spores. In the present study, trials with aqueous spore suspensions of A. zinniae showed that centrifuging at 1,450 r.p.m. for 10 minutes was sufficient to deposit over 85% of the conidia.

Materials and Methods.

Method 1. A two gram sample from each of four naturally infected sedlines of Zinnia elegans was used for each test. The seed was placed in a 100 ml. flask wit 25 ml. of sterile distilled water and shaken vigorously for 5 minutes. The wash water was decanted into four hard glass test tubes and centrifuged at 1,450 r.p.m. for 10 minutes. Most of the supernatent in each tube was discarded, and the sediment poured into a test tube, the total volume being made up to 10 ml. with sterile distilled water. A drop of this concentrate was examined under a microscope to confirm the presence of spores of A. zinniae. The spore suspension was sprayed onto 10 healthy zinnia plants and these, along with three control plants which had been sprayed with sterile distilled water, were placed in a high humidity cabinet in the glasshouse. After 48 hours the plants were removed to the glasshouse bench. Disease manifestation was recorded 10 days after inoculation, the number of leaves showing lesioning being counted. Reisolations were made by removing diseased leaves to the laboratory, placing them on slides in petri plates lined with moist filter paper for 48 hours at 28°C, transferring single spores to slopes of PDA.

Tests were carried out using this seed washing method for determining conidial viability at approximately 3 week intervals.

Method 2.

During the winter it was found difficult to maintain sufficient plants for spore longevity studies using Method 1. Accordingly, a laboratory method was used for demonstrating the viability of spores in seed washings when sufficient plants were not available. The method used limited the risk of cultural contamination when using an impure source of spores such as from the

seed surface. The method involved placing the spore suspension between the agar and the glass bottom of the petri plate by inverting the agar, as suggested by Sleeth (1945). By this method the spores could be readily found and observed through the bottom of the plate without being obscured by mycelial or bacterial contaminants. Fungi often grew through the agar and could be isolated from the agar surface.

Seed washings from each of the four lines under test were centrifuged and resuspended in 5 ml. sterile distilled water. The resultant concentrate was drawn into a capillary pipette and 0.1 ml. drops of the spore suspension placed in each petri plate on the surface of the prune agar used, 5 drops per plate.

The entire agar disc was then inverted, using a broad spatula. The raised rim at the edge of the plate was trimmed off so that the agar surface could adhere to the bottom of the plate without entrapment of air. Plates were incubated at 28°C. As soon as colonies were visible they were examined through the bottom of the plate under a microscope. Colonies developing from single spores of A. zinniae were easily identified. Pure culture transfers from the top surface of the agar could often be made by taking shallow agar plugs. The number of spores which had germinated 12 hours after inoculation was determined and recorded as a percentage. Usually 50 spores were observed per plate and an average percentage figure of the spores which had germinated used as a measure of viability of conidia from seed washings.

Viability tests on conidia associated with each of the four seedlines under study were carried out at approximately 3 week intervals.

Results and Discussion.

Both of the methods employed to determine the viability of conidia from seed washings proved effective. The method involving spraying seed washings on to healthy plants (Method 1) was used when possible and the laboratory method (Method 2) used only in cases when insufficient plants were available for glasshouse tests. Results of the tests are presented in Table 22. Percentage figures are recorded to the nearest whole number.

TABLE 22. SURVIVAL OF LOOSE CONIDIA ADHERING TO THE SEED SURFACE OF NATURALLY INFECTED LINES OF ZINNIA ELEGANS.

Date	Seedline of 1962 seed used. A	B	C	D	Approx. age of seed. (months)	Method used for Viability Test.
27.2.63	100	100	92	98	16	1
4.3.63	100	98	88	100	16.5	1
3.4.63	84	88	89	92	17.5	2
1.5.63	72	84	60	88	18.5	1
21.5.63	44	40	41	62	19	2
14.6.63	31	55	61[a]	22	20	1
1. 7.63	16	26	24	20	20.5	1
24.7.63	43	43	37	36	21	2
20.8.63	21	29	29	25	22	2
11.9.63	17	34	26	27	23	1
1.11.63	19	29	23	39	24.5	1
3.12.63	22	NR	26	23	25.5	1
7.1.64	19	NR	17	14	27	1
29.1.64	7	NR	19	16	27.5	1
26.2.64	2	NR	8	5	28	1
23.3.64	NR	NR	4	5	29	1
27.4.64	NR	NR	0	0	30	1
22.5.64	NR	NR	0	0	31	2

NR = Test discontinued due to lack of seed.

[a] = 1 control plant showed 2 leaves infected.

These results show that loose conidia of A. zinniae are capable of remaining viable on the surface of zinnia seed for up to $2\frac{1}{2}$ years from harvest. Thus they may be regarded as a potential source of inoculum for the infection of seedlings arising from contaminated seed, even when such seed is held for 2 years before sowing.

There did not seem to be any marked difference in results as recorded by Methods 1 and 2. In cases where Method 1 was used, there was quite high variation in the percentage of disease expression between successive tests. This was possibly due to unfavourable temperatures in the glasshouse following inoculation, since no control of temperature was made during each test.

Conclusion.

From these results it is concluded that conidia on the surface of seed can remain viable for up to 29 months from harvest, and are capable of causing infection and development of disease symptoms when sprayed onto healthy plants in the glasshouse. This study therefore implicates loose spores adhering to the seed surface of imported lines of Zinnia elegans as a source of primary inoculum for seedling infection. At no stage during this trial was there any evidence of a spore dormancy phase or any sign of mycelium production associated with spores of A. zinniae adhering to the seed surface.

(ii) Survival as mycelium within the seed.

In order to determine whether vegetative mycelium of A. zinniae established in seed tissue was capable of remaining viable over long periods, further studies were carried out. The same four naturally diseased seedlines used in part (i) were used in this study. These lines had produced varying percentages of diseased seedlings ranging from 12,- to 25%, ten days from sowing. The seed was dusted to kill any conidia adhering to the seed surface, so that when seed was germinated on moist blotters any diseased cotyledons present could have arisen only from mycelium of A. zinniae within the seed.

Materials and Methods.

A sample of 200 seeds from each line was placed in a 100 ml. beaker and shaken with Fernasan dust for 3 minutes to obtain complete seed coverage. A volume of dust approximately equal to the volume of one seed of the variety being tested was used. The seed was then tipped onto a 1mm mesh screen and shaken to remove excess dust. The seed was germinated on moist blotters in a Copenhagen

tank at 25°C. After 7 days, 20 lesioned cotyledons were removed, washed in running tap water for 6 hours and plated aseptically to prune agar. Plates were incubated at 28°C for four days. Fungal colonies arising from plated tissue were cut out with a 5mm. corkborer and the agar circles so formed incubated on the underside of a petri plate lid for 48 hours at room temperature. Positive identification of colonies of A. zinniae was made by reference to spore characters

Tests were carried out at approximately monthly intervals.

Results and Discussion.

Results on the length of time A. zinniae can remain viable as vegetative mycelium within the seed are recorded in Table 23. Results are presented as the number of successful reisolations obtained from 20 lesioned cotyledons plated.

TABLE 23. VIABILITY OF A. ZINNIAE AS MYCELIUM WITHIN THE SEED.

Date of Test.	Age of Seed - months.	Seedline Used. A	B	C	D
20.2.63	16	12	14	8	16
20.3.63	17	10	12	6	12
16.4.63	18	12	13	6	13
18.5.63	19	8	12	7	14
28.6.63	20	9	15	7	12
15.7.63	21	14	10	6	9
20.8.63	22	10	12	8	10
11.9.63	23	9	11	7	8[a]
1.11.63	25	9	10	7	13
3.12.63	26	11	NR	11	14
8.1.64	27	12		8	12
26.2.64	28	10		7	10
20.3.64	29	NR		4	13
27.4.64	30			9	14
23.5.64	31			8	16
20.6.64	32			8	9
16.7.64	33			6	10
13.8.64	34			8	8
16.9.64	35			6	12
20.10.64	36			7	12
14.11.64	37			6	13

Trial discontinued.

[a] = Only 12 lesioned cotyledons plated.

NR = Trial with a particular seedline discontinued due to insufficient seed for further testing.

Although tests were discontinued when the seed was approximately 3 years old, this trial shows that vegetative mycelium of A. zinniae borne within the

seed has a greater survival ability than loose conidia contaminating the seed surface.

Conclusion.

In the present trial A. zinniae was shown to be capable of surviving as internal mycelium in seed for over 3 years. This would indicate that storage of zinnia seed for at least 3 years from harvest is of no practical value in eliminating the danger of spread of viable inoculum by way of the seed.

From this trial it is concluded that loose conidia adhering to the seed-coat and vegetative mycelium of A. zinniae within the seed are both of great importance as sources of primary inoculum for the infection of seedlings arising from seed which is not more than two years old. Although surface borne conidia have been shown to remain viable for about $2\frac{1}{2}$ years, internal mycelium of A. zinniae has been demonstrated as being capable of remaining viable and of causing lesions on seedlings arising from seed at least three years from harvest.

(b) SURVIVAL IN DRIED HOST TISSUE.

Studies were conducted to determine the ability of A. zinniae to remain viable in diseased zinnia leaves stored under glasshouse conditions.

Materials and Methods.

Lesioned tissues used in this experiment were obtained from a naturally infected crop of variety Giant Dahlia Flowered on 25th February, 1963. The leaves were spread out on a bench in the glasshouse to dry for 7 days and then placed in a cardboard box for storage. At intervals, three leaves were removed to the laboratory and placed on slides in petri plates lined with moist filter paper. The plates were held at 28°C. Spore production was observed after 30 hours, and single spores transferred to slopes of PDA to prove viability.

Results and Discussion.

Results of observations on relative spore production after high humidity treatment of diseased leaves for 30 hours at 28°C are recorded in Table 24.

TABLE 24. SURVIVAL OF A ZINNIAE AS MYCELIUM IN DRIED HOST TISSUE.

Date of Test.	Length of Storage. in days.	Relative Spore Production.	
25.2.62	0	5	Very Heavy Sporulation.
10.3.62	13	5	
24.3.62	27	5	
9.4.62	43	4	Wide variation in spore numbers produced from lesions on the same leaf.
30.4.62	64	5	
17.5.62	81	5	
4.6.62	109	5	
30.6.62	135	4	
14.7.62	149	5	Spore production variable on different leaves and on different regions of the same leaf.
29.7.62	164	4[a]	
22.8.62	188	4	
16.9.62	213	3	
12.10.62	239	3	Spore production moderate.
7.11.62	265	4	
6.12.62	294	3	
23.12.62	311	2	Spore production sparse and very variable both between leaves and on different regions of the same leaf.
16.1.63	335	2	
25.2.63	375	2	
Observations discontinued.			

a = Incubated at 28°C under high humidity for 24 hours only.

Relative Spore Production represented on an arbitrary scale 0 to 5:

0 = Nil
1 = Very sparse.
2 = Sparse
3 = Moderate
4 = Heavy
5 = Very heavy.

The fungus retained its viability for at least 12 months in dried leaves of diseased zinnia plants. Conidia which were proved viable on slopes of PDA were often subcultured to PDA plates and used to provide cultures for the preparation of spore inoculum for host range pathogenicity studies. No loss of pathogenicity was observed during this trial, although the numbers of conidia produced declined markedly over the test period.

Conclusion.

This experiment demonstrates the ability of A. zinniae to remain viable in dried host tissue for at least 12 months. This period is considered to be sufficient for spores produced from such tissue to be of some importance as a potential source of inoculum for the infection of new season's crops under conditions where diseased zinnia leaves are allowed to remain over the winter months under glass by inadequate care to sanitation and glasshouse hygiene.

(c) SURVIVAL ON ALTERNATE HOSTS.

Experiments were carried out to determine whether A. zinniae could survive by means of alternate hosts in the absence of zinnia plants. Several plant species were found in the field to possess lesions macroscopically identical to those produced by A. zinniae on zinnias. Each isolate from such plants was inoculated to zinnias after a pure culture had been obtained from single conidia. Results are recorded in Table 25.

TABLE 25. PATHOGENICITY OF *A. ZINNIAE* ISOLATED FROM NATURALLY INFECTED HOST SPECIES OTHER THAN ZINNIA.

Host.	Botanical Name.	Pathogenicity to Zinnia	Pathogenicity to Host.	Comments.	
Ageratum	*Ageratum conyzoides*	+	+		1
Aster	*Callistephis Chinenis*	+	+	Weakly pathogenic to both Zinnia and host.	1
Black nightshade	*Solanum nigrum*	+	+	Older leaves more susceptible	1
Calendula	*Calendula officinalis*	+	+	Strongly pathogenic to zinnia	1
Cineraria	*Senecio cruentus*	+	-		1
Cornflower	*Centaurea cyanus*	+ W +	+ -	Weakly pathogenic.	2
Hydrangea	*Hydrangea macrophylla*	+	+	Weakly pathogenic to zinnia	1
Lettuce	*Lactuca sativa*	-	-		2
Lupin	*Lupinus polyphyllus*	+	+ weak		2
Marigold	*Tagetes erecta*	+	+	Strongly pathogenic to zinnia	1
Sunflower	*Helianthus annuus*	+	+		1
Tomato	*Lycopersicon esculentum*	-	+	Not *A. zinniae* (*A. solani*?)	2

KEY:

1 = Isolate identical to *A. zinniae* in cultural and spore characters.

2 = Isolate not identical to *A. zinniae* in cultural and spore character.

W = Plant wounded prior to inoculation.

\+ = Inoculation successful.

\- = Inoculation unsuccessful.

In view of the restricted spread of conidia of *A. zinniae* to relatively short distances, and the inconsistency of association of many host plants with areas of zinnias, the possibility that the majority of these species may serve as alternate hosts for *A. zinniae* in the absence of zinnia plants seems remote.

Because the majority of the alternate hosts listed in Table 25 are annuals, they cannot be considered to be of importance as hosts on which A. zinniae can perennate in the absence of zinnia plants. Some perennial species on the other hand, (e.g. Hydrangea), may be of some importance as overwintering hosts of A. zinniae if they are present in close proximity to areas consistently planted in zinnias. The possibility also exists however that flower-stalks of some of the annual species found to be naturally infected with A. zinniae, (e.g. Tagetes erecta and Calendula officinalis), may survive through the winter in sheltered situations in home gardens. These hosts may provide a method by which A. zinniae can overwinter, and may serve as a source of primary inoculum for infection of nearby zinnias planted the following spring. This is supported by the successful reisolation of A. zinniae from lesions on the stems and old flowerheads of marigold plants (Tagetes erecta) in home gardens in Palmerston North during the winter, and the association of A. zinniae with such plants of T. erecta throughout the year.

Conclusion.

Because of the localised spread of A. zinniae spores and the inconsistent association of demonstrated susceptible hosts with areas of outdoor plantings of zinnias in the spring, the use of alternate hosts as an overwintering mechanism for A. zinniae is considered to be of minor importance.

(d) SURVIVAL AS FREE CONIDIA.

In nature, conidia of pathogenic fungi are subjected to a variety of adverse conditions which may result in death of the spores before they can successfully attack a susceptible host (Goos 1962). Experiments were accordingly carried out to determine the longevity of conidia of A. zinniae stored in a dry state on a non-nutritive substrate.

(i) Conidial survival was investigated on sterile glass coverslips. The spores used in this study were obtained from the surface of a 10 day old culture of A. zinniae on PDA, which had been induced to sporulate by exposure to ultraviolet

light for 1 minute. Spores were transferred from the surface of the culture onto sterile coverslips in petri plates using a fine camelhair brush. The petri plates were placed under a bell-jar at room temperature. Tests on conidial viability under such conditions were made each day by transferring single spores to slopes of PDA. which were incubated at 28°C.

Conidia stored in a dry state under relatively constant humidity, with fluctuating conditions of light and temperature, remained viable for 33 days.

(ii) Conidial survival was studied on sterile glass coverslips in petri plates stored outside. Spores were transferred from a sporulating culture to coverslips by the method described above. The petri plates were placed in an exposed location outdoors and an empty tin inverted over the top to prevent entry of rainwater. Spore viability was determined by removing a coverslip to the laboratory every 4 days and transferring single spores to slopes of PDA.

Conidia stored outside under natural conditions of humidity, light, and temperature fluctuations remained viable for 28 days but not 32 days.

(iii) Conidial survival was studied as in (ii) above, except that spores were placed on sterile coverslips at high spore density, conidia being aggregated together in clumps. Viability was determined by removing a coverslip at frequent intervals and transferring dried clumps of spores to slopes of PDA.

Under such conditions conidia stored outside in clumps on sterile coverslips survived 185 days but not 189 days.

(iv) Spores of A. zinniae were stored in a dry condition on sterile coverslips in petri plates at room temperature. The plates were placed on the sill of a north window and in this situation were exposed to full sunlight during part of the day. Tests of viability were made at 4 day intervals by transferring single spores to slopes of PDA.

Under such conditions of storage, conidia retained their viability for 48 days.

(v) To determine whether conidia of A. zinniae present on a non-nutritive substrate in large clumps could survive longer periods than conidia stored in

thin films, a spore suspension (230,000 spores in 1ml. sterile distilled water) was poured into the bottom of a petri plate. Natural evaporation was allowed to take place at room temperature. The plate, containing clumps of dry spores was held at 28°C, and every 4 days a clump of conidia was removed and transferred to a plate of PDA to prove their viability. Mycelial growth and the ability of the resultant culture to produce spores were noted.

Under such conditions of constant temperature conidia in clumps were capable of survival for at least 460 days, at the end of which time viability tests were discontinued.

(vi) Conidial survival on glass coverslips stored at a constant temperature was also studied. Thin films of loose spores were placed on sterile coverslips which were then placed in petri plates and held at 28°C. Spore viability was determined by transferring single spores to plates of PDA.

The conidia remained viable for 136 days, but lost the ability to sporulate on culture media after storage for 124 days.

Discussion.

As a rule, sexual spores of fungi generally survive longer than asexual spores. This is especially true where the former are contained within some form of protective fruiting body. The conidia of some imperfect fungi, however, may survive very long periods. The asexual spores of some imperfect fungi have therefore taken over the function of carrying the organism over long periods of unfavourable conditions.

The conidia of A. zinniae are remarkably resistant to loss of viability, although external conditions may influence longevity, possibly by their effect on spore metabolism, on the rate of disappearance of reserve materials, or on cofactors essential for germination.

The results of the above experiments on conidial survival of A. zinniae are summarised as follows:

Test.	Spore Density.	Storage Conditions. Temperature.	Humidity.	Light intensity.	Survival capacity in days.
i.	Low	F (room temp)	C	F	33
ii.	Low	F (outdoors)	F	F	28
iii.	High	F (outdoors)	F	F	185
iv.	Low	F (room temp)	F	F	48
v.	High	C (28°C)	F	L	460 +
vi.	Low	C (28°C)	F	L	136

F = Fluctuating, C = Constant, L = Low.

From these results it appears that temperature conditions are of great importance in determining spore longevity. Light intensity does not appear to affect survival to any great extent, but humidity fluctuations may have some effect.

Conidia of A. zinniae stored at low spore density on coverslips at room temperature (test iv.) survived 42% longer than conidia stored under the same external conditions of fluctuating temperature, humidity and light intensity outdoors (test ii.). Similarly, conidia stored on coverslips at low spore density at 28°C (test vi.) survived nearly 4 times longer than spores at low spore density held outside, (test ii.). These results indicate that low temperatures have a pronounced effect in decreasing spore longevity when conidia are present at low spore densities in a dry state.

External humidity also appears to be of importance in influencing spore longevity of A. zinniae. Conidia stored at low spore density at room temperature and under conditions of fluctuating humidity and light (test iv.) survived 15 days (31%) longer than spores from the same culture stored under similar conditions of temperature and light, but held at constant humidity (test i.).

In 1916 Dasteur (Goos 1962) reported that conidia of Gloeosporium musarum Cooke and Massee, a common pathogen of bananas, survived dessication for as long as 9 months when the spores were present in clumps. On the other hand, thin films of spores were quickly killed by drying. In the present study spore density was

shown to have a great effect on the survival ability of spores of A. zinniae. Spores stored at high spore density at 28°C, under conditions of fluctuating humidity and low light intensity (test v.) remained viable at least 10 months (324 days) longer than conidia stored under the same conditions but at low spore density (test vi.). Similarly, conidia stored in aggregated masses outdoors (test iii.) remained viable 5 months (157 days) longer than spores stored under the same conditions but present at low density (test ii.). This indicates that longevity of spores of A. zinniae is greatly increased when they are aggregated into clumps rather than being present as individual separated spores. This may be explained by spores in clumps being more resistant to dessication and external conditions of temperature and humidity than spores which are present in a thin film.

Conclusions.

Conidia of A. zinniae survived a non-nutritive substrate for at least 15 months under conditions of high temperature (28°C) and fluctuating humidity, and when spores were present in an aggregated mass. Conidia stored under similar conditions in low spore densities retained their viability for only 4½ months. This effect is postulated as being due to greater resistance of spores in clumps to dessication of bound water within the protoplasm of the spore. Under conditions of lower temperature, whether in the laboratory or outside, spores survived a maximum of 1.6 months.

This study indicates that conidia of A. zinniae survive longest on a non-nutritive medium at high temperatures. Thus it is concluded that spores present in clumps on the walls and benches of heated glasshouses may retain their viability long enough to be of importance as a source of primary inoculum for infection of zinnia seedlings planted under glass the following season. Survival as loose spores on objects outside is not considered to be of any importance as a means by which the pathogen may overwinter.

(e) SURVIVAL IN THE SOIL.

To determine whether inoculum of A. zinniae could remain viable in the soil in the absence of zinnia plants, a 100 gram sample of soil was collected from an outside bed which had contained a heavily diseased crop of zinnias of variety Giant Dahlia Flowered, 12 months previously. The soil was mixed into a slurry with sterile distilled water and painted onto the leaves of healthy zinnia plants in the glasshouse. Plants were also painted with a slurry of autoclaved soil and sterile distilled water as controls. After 24 hours in a high humidity cabinet plants were removed to the glasshouse bench. After 7 days the soil was removed from the leaves with a fine spray of tap water and any leaves which had developed lesions were removed to the laboratory. Conidia of A. zinniae were produced from lesions by high humidity treatment of diseased leaves in petri plates lined with moist filter paper and held at 28°C for 48 hrs. Single spores were removed to plates of PDA to confirm viability of the fungus. Isolates were induced to sporulate by exposure to ultraviolet light. A suspension was made from spores formed by each isolate from 'painted' leaves and inoculated to healthy zinnia plants. A spore suspension prepared from a stock culture of A. zinniae originally obtained from diseased zinnia leaves, was also inoculated to healthy plants as a control. Lesions appeared on the leaves of inoculated plants within 72 hours and successful reisolations were made to PDA. The plants inoculated with isolates obtained from painted leaves showed lesions macroscopically identical with those produced on plants inoculated with spores of A. zinniae from the stock culture. Cultural characteristics and spore morphology were also identical.

This experiment proved the ability of inoculum of A. zinniae to remain viable in the soil for at least 12 months between zinnia crops. Further study was therefore carried out to determine the form in which inoculum persists in the soil and to determine whether the pathogen could survive in the soil as:

(i) Mycelium associated with soil particles.
(ii) Mycelium associated with soil organic matter.
(iii) Mycelium in buried crop debris.
(iv) Loose conidia.

(i) Mycelium associated with soil particles.

Hyphal isolation from soil particles.

The technique of hyphal isolation from soil was developed by Marcup (1955) who observed that when a soil suspension was prepared, many of the fungal hyphae remained in association with the heavier soil particles in the residue. Removal of the fine suspended material from the residue of a sample of soil permitted visual examination of the residue for the presence of individual hyphae or hyphal masses which were then removed and grown on agar.

Two soil samples were used in this experiment:

1. A sample obtained from an outside bed which had contained a heavily diseased crop of zinnias 12 months previously.

2. A sample of sterilised soil which had been artificially inoculated with mycelium of A. zinniae 3 months previously. To obtain mycelial fragments for artificial inoculation of the soil a colony of A. zinniae was grown in each of four 500ml. flasks of liquid culture of FDA at room temperature. During incubation the flasks were shaken continuously on a mechanical shaker. This allowed the production of hollow spherical colonies of A. zinniae which could readily be removed from the media and dried. Colonies produced in this way were free from spores. When the colonies were approximately 3 cm. in diameter (after 14 day's growth) they were removed from the flasks and allowed to dry between two sheets of sterile blotting paper for 48 hours at room temperature. The colonies were then placed in a waring blender with 50 ml. of distilled water and finely macerated at 2,500 r.p.m. for 10 minutes, to produce a slurry of fine mycelial fragments which was thoroughly mixed with 1 lb. of sterilised soil and stored at room temperature in a glass jar.

Approximately 1.8 - 2.0 gm. from each of the two soil samples was placed in a beaker and disintegrated with a jet of sterile water. In both cases the soil was allowed to settle and the supernatent poured off. More water was added and again the supernatent poured off. This process was repeated until the liquid

became clear on standing for 2 minutes. The soil particles in the residue were placed in a petri plate and distributed in a small quantity of sterile water, then broken apart under the microscope. Individual hyphae were removed using a finely pointed pair of forceps. These hyphae were plated to prune agar and the plates held at 28°C.

Tips of the growing hyphae considered to be similar to those of A. zinniae were cut out in agar blocks and transferred to fresh media for subsequent growth and identification.

Of the 30 fungal hyphae recovered from soil particles and plated to agar three Alternaria isolates were obtained of which two were later shown to be A. zinniae. These two isolates proved pathogenic to zinnias. The two successful isolations of A. zinniae from soil particles were recovered from artificially inoculated soil. No reisolations of A. zinniae from hyphal fragements were recorded from the sample of soil from an outdoor bed which had contained a heavily diseased crop of zinnias the previous season.

From these results it is concluded that survival of A. zinniae by means of vegetative mycelium living saprophytically in soil particles is of no importance as a method of overwintering of the pathogen in the absence of a susceptible host crop.

(ii.) Mycelium associated with soil organic matter.

Hyphal isolation from organic matter in the soil.

The method employed to determine whether A. zinniae could survive as vegetative mycelium associated with organic matter in the soil was essentially the same as described above except that the organic matter was recovered from the soil by shaking with water and removed the material which floated to the surface.

A sample of 2 gm. of crumbed soil was obtained from both artificially inoculated soil and from soil recovered from an area which had contained a heavily diseased crop of zinnias the previous season.

Each sample was broken down with a fine jet of sterile water and the organic matter recovered by flotation. This material, after several washes in sterile water was examined in the same manner as in (i.) above.

Twenty fragments of hyphae (ten per sample) were recovered from the organic matter. These were plated to prune agar and later subcultured by transferring agar squares to fresh plates for growth and identification.

From the twenty samples of hyphae recovered from organic matter in the soil, ten from each soil sample, and plated to agar, nine cultures of Alternaria were obtained of which 4 were A. zinniae and at least 4 of the remainder A. tenuis. The four cultures of A. zinniae were all pathogenic to zinnia plants although there was a marked variation in growth rate on culture media between isolates. The hyphae of A. zinniae recovered from soil organic matter all came from artificially inoculated soil, no successful reisolations of the fungus from hyphae being made from soil in which diseased zinnias had been grown.

Accordingly it is concluded that A. zinniae cannot survive as vegetative mycelium associated with organic matter in the soil between seasons in the absence of a susceptible crop.

On the basis of the above experiments survival of A. zinniae as vegetative mycelium in the soil is not considered to be important as a means of perennation of the pathogen in the absence of a susceptible host crop.

(iii.) Survival in infected plant debris buried in the soil.

The possibility that A. zinniae may overwinter as vegetative mycelium within plant tissue from a previous season's crop has been considered by some workers as a potential method of survival of the pathogen in the absence of a susceptible host crop. Dimock & Osborn (1943) record that "it was demonstrated that the fungus may live at least one winter on plant debris in the soil or on its surface" Several other authors (Page 1942, Neergaard 1945, Forsberg 1946, Baker & Davis 195 and Kreitlow 1961) state that sanitation by burning crop refuse and cleaning beds

in the autumn should be practised to prevent carryover of the fungus from crop to crop.

Trials were conducted to determine whether A. zinniae can remain viable in buried crop debris for a sufficient length of time for this method of overwintering to be important in providing primary inoculum for new season's infection of zinnia plants.

Heavily diseased leaves and stems were collected from a naturally infected crop of zinnias of variety Giant Dahlia Flowered. Diseased tissue was placed in open petri plated and 'layered' in each of two jars of non-sterilised soil using six plates per jar. The purpose of placing diseased leaves and stems in petri plates was to facilitate the recovery of the plant material for further study. One jar containing diseased tissue in petri plates buried in the soil was stored at room temperature in the laboratory. The other jar was placed outdoors in an exposed location. At approximately 14 day intervals attempts were made to reisolate the pathogen from diseased tissue by both of the following methods:

(a) Diseased tissue was recovered from each jar and placed under high humidity in petri plates lined with moist filter paper in the laboratory. Plates were held at 28°C for 48 hours. Single spores arising from diseased leaves and stems were transferred to plates of PDA to prove viability. Plates were incubated at 28°C and colony diameter recorded at 10 days.

(b) Tissue pieces containing lesions were recovered from the soil and surface sterilised by immersion in 0.1% mercuric chloride solution for 2 minutes. These tissue pieces were then thoroughly rinsed in sterile distilled water and plated aseptically in prune agar. Plates were held at 28°C. Diameter of fungal colonies arising from plated tissue was recorded at 10 days.

The results of tests to recover and prove viability of A. zinniae from debri of zinnia plants buried in the soil are presented in Table 26.

TABLE 26. VIABILITY OF *A. ZINNIAE* FROM DEBRIS BURIED IN THE SOIL.

Date of Test.	Length of Storage in days.	METHOD 1. Colony diam. - 10 days.		METHOD 2. Colony diam. - 10 day	
		R.T.	E.L.	R.T.	E.L.
20.2.63	0	86	87	88	87
28.2.63	8	86	90	86	86
7.3.63	15	85	90	86	87
14.3.63	22	81	86	87	82
29.3.63	37	80	82	85	82
10.4.63	49	84	83	83	80
26.4.63	65	81	Nil	80	80
12.5.63	81	80	Nil	78	Nil
24.5.63	93	Nil	Nil	80	Nil
16.6.63	116	Nil	Nil	Nil	Nil
2.8.63	163	Nil	Nil	Nil	Nil
7.10.63	229	Nil	Nil	Nil	Nil
8.11.63	261	Nil	Nil	70	Nil
6.12.63	289	78	Nil	70	Nil
10.1.64	324	66	Nil	62	Nil
12.2.64	357	62	Nil	58	Nil
14.3.64	388	Nil	Nil	56	Nil
26.3.64	400	Nil	Nil	Nil	Nil

R.T. = Storage at room temperature.

E.L. = Storage in an exposed location outside.

Nil = Indicates reisolation unsuccessful.

These results demonstrate the ability of A. zinniae to retain its viability in diseased tissue buried in the soil for varying lengths of time dependent on environmental conditions.

The pathogen was successfully reisolated from plant debris buried in soil held at room temperature for 388 days, but from material stored in soil outdoors for only 65 days. This trial therefore indicates that the survival of A. zinniae in diseased host tissue buried in the soil outside cannot be important as a potential source of primary inoculum for infection of new season's zinnia plants.

The main external factor influencing survival of A. zinniae in buried crop debris appears to be temperature. The pathogen in debris buried outdoors showed a complete loss of viability in late April, coincident with the first winter frost. In buried debris stored indoors at room temperature the fungus entered a dormancy phase over the winter months and was successfully reisolated from diseased tissue in the following spring. This dormancy phase appeared to be induced by the onset of frosts and low diurnal temperatures. No dormancy phase was observed in A. zinniae in crop debris buried outdoors, attempts to reisolate the fungus from such tissue being unsuccessful.

The progressive decline in vigour of A. zinniae reisolated from buried crop debris is reflected in the following:-

(a) A reduction in sporulation capacity.

(b) A reduced growth rate of single spores when plated in agar.

(c) Complete loss of sporulating ability.

(d) A reduction in colony diameter of surface sterilised tissue plated to agar.

(e) Total loss of viability, in that the fungus could not be reisolated from plated tissue.

From the results obtained in this trial it is concluded that the over-wintering of A. zinniae in debris of infected plants buried in the soil is of no importance. In outdoor plantings of zinnias the pathogen cannot survive between crops in diseased tissue in the soil. Under glass the fungus has been shown to

be capable of surviving for at least one year in diseased debris of zinnia plants buried in the soil. This method of overwintering, however, would be of no importance where zinnias are grown in sterilised soil and where previously used soil in nurseries is discarded,or re-sterilised before use.

(iv) Survival as free conidia in the soil.

The ability of spores of A. zinniae to survive for long periods on non-nutritive substrate has been described previously. On the basis of this work and in view of the fact that the pathogen has been reported as overwintering in the soil, "the usual 2-3 year rotation being insufficient to control the disease" (Baker & Davis 1950), studies were carried out to determine the longevity of loose conidia of A. zinniae buried in the soil. This trialwas carried out in two parts:

(a) In artificially inoculated soil in the laboratory, and

(b) In naturally infected soil from the field.

(a) Survival of conidia of A. zinniae in artificially inoculated soil.

Samples of sterilised and unsterilised soil were infested with a spore suspension containing 100,000 spores/ml. Sufficient water was then added to each sample to bring the soil to its moisture equivalent. Inoculated soil samples were stored in open glass jars at room temperature in the laboratory. Tests to assess the viability of spores from each sample were conducted at approximately 3 week intervals. The two methods used for this purpose were:-

Flotation method.

This technique was essentially the same as described by Ledingham & Chinn (1955) for the recovery of spores of Helminthosporium sativum from soil. This method utilises surface tension phenomena and appears well suited for the recovery of large sized spores from soil.

Approximately 5 gm. of soil from each sample was screened on a $1mm^2$ mesh seive to remove large pieces of debris. Each sample was placed in a test tube and a surplus of sterile distilled water added. The soil and water were shaken and 3 ml. glycerine added to each tube. The tubes were agitated vigorously for

3 minutes and placed in a rack in a vertical position. After 20 minutes most of the soil had settled to the bottom and an emulsion collected at the surface. Excess soil particles held in the emulsion were settled out by addition of a small amount of sodium chloride. The emulsion, approximately 3 ml, was pipetted off and placed in a sterile test tube and distilled water added to make the total volume up to 10 ml. The presence of spores in the solution was verified by microscopic examination. In most cases percentage recovery of spores was very high.

The spore suspensions obtained by the use of this flotation method were atomised onto the leaves of healthy zinnia plants in the glasshouse. Two plants were sprayed with sterile distilled water as controls.

Leaf Painting Method.

From each soil sample approximately 1 gm. was removed at 3 weekly intervals, wetted to a semi-liquid consistency with sterila distilled water and brushed onto the leaves of healthy zinnia plants. The leaves of two plants were painted with a slurry of sterilised soil and water as controls.

Two sets of plants were inoculated using each of these methods, held in a high humidity cabinet in the glasshouse for 48 hours and then removed to the glasshouse bench. Seven days after inoculation the leaves of the plants which had been painted with soil were sprayed with a fine jet of water to remove adhering particles. Counts of the number of leaves which had developed lesions were made 10 days after inoculation. Reisolations were made by removing diseased leaves to the laboratory and subjecting them to high humidity conditions for 48 hours at 28°C. The resultant spores were proved viable by transferring single spores to sloped of PDA.

Some initial difficulty was experienced in determining the number of infected leaves of plants inoculated with spores obtained by the flotation method because the burning effect of the glycerine on the leaves tended to mask symptom expression by the fungus. By reducing the amount of glycerine used in

the flotation process from 3 ml. to 1 ml. leaf burning was markedly reduced.

The use of the soil painting method, while it gave reasonable results, was discontinued during the winter when the supply of plants was inadequate.

Results of inoculation experiments to determine the longevity of loose conidia of A. zinniae buried in the soil are presented in Table 27.

TABLE 27. LONGEVITY OF LOOSE SPORES OF A. ZINNIAE IN ARTIFICIALLY INOCULATED SOIL.

Date of test.	Length of Storage, in days.	% Infection at 10 days. Method 1.		% Infection at 10 days. Method 2.	
		Ster. soil.	Unster.soil.	Ster. soil.	Unster.soil.
28.2.63	0	100	100	92	90
2.3.63	2	100	100	83	76
7.3.63	7	96	91	64	52
21.3.63	21	89	87	76	76
10.4.63	41	100	100	80	84
7.5.63	68	84	87	84	88
27.5.63	88	90	85	76	81
12.6.63	104	87	81	NR	NR
5.7.63	127	82	88	NR	NR
24.7.63	146	76	80	NR	NR
16.8.63	169	90	96	NR	NR
12.9.63	196	94	96	NR	NR
27.10.63	241	92	86	NR	NR
3.12.63	278	46	38	NR	NR
7.1.64	313	53	50	NR	NR
29.1.64	335	42	55	NR	NR
26.2.64	363	41	44	NR	NR
6.4.64	372	52	40	32	46
27.4.64	393	46	49	31	39
20.6.64	447	19	16	22	27
29.6.64	456	4	9	5	7
14.7.46	471	2	0	0	3
23.7.64	480	0	0	0	0

Ster. = Sterilised. Unster. = Unsterilised. NR = Not recorded.

These results show that loose conidia are capable of surviving in both sterilised and non-sterilised soil for periods more than sufficient to allow their persistance in a viable condition between successive crops. At no stage during this trial was there any evidence of spore germination in the soil, nor was there any sign of mycelium which may have arisen from the germination of conidia in the soil.

The percentage infection of plants inoculated with spores recovered from the soil varied quite considerably between tests. This variation may have been due to unfavourable temperature during disease development in the glasshouse. Results from counts of infected leaves produced by atomising spores recovered by the flotation method onto disease-free plants tended to be more uniform between tests than the results obtained by the soil painting method.

Conidia of A. zinniae inoculated to both sterilised and non-sterilised samples of soil were shown to retain their viability for at least 15 months. Thus loose conidia in the soil must be considered to be an important source of primary inoculum for the infection of a new season's crop. It is postulated that the methods by which conidia of A. zinniae in the soil come into contact with plants in a new season's crop could be by,

1. Infection of seedlings during seed germination.
2. Throwing of soil containing spores onto the plants during cultivation in the crop to control weeds.
3. Splashing of soil onto plants during a heavy rain.

(b) Survival of conidia of A. zinniae in naturally infected soil.

Studies were carried out to determine the ability of loose conidia to survive in the soil. Samples were obtained from each of 5 locations in which zinnias had been planted. The length of time since the soil had contained zinnias varied from 14 months in the case of an outdoor bed, to two weeks for soil from a nursery box from which seedlings had just been transplanted.

All samples were seived through a 1 mm^2 mesh to remove large debris. Tests to assess the viability of spores present in each soil sample were made by the two

methods described in (a). Where the flotation method was used, 3 additional plants per sample were sprayed with sterile distilled water as controls. Where the soil painting method was employed the leaves of 3 plants per sample were painted with a slurry of autoclaved soil and water as controls.

Counts of the number of leaves showing lesioning were made 10 days after inoculation. Reisolations were made by removing diseased leaves and placing them under high humidity conditions at 28°C for 48 hours. Single spores were then transferred to slopes of PDA to prove viability.

The results of infection counts obtained on leaves 10 days after inoculation are recorded in Table 28.

TABLE 28. SURVIVAL OF FREE CONIDIA IN NATURALLY INFECTED SOIL.

Source of Sample.	Approx. time since zinnias present. Months.	Flotation Method. No. leaves Inoculated.	% Infection.	Painting Method. No. leaves Inoculated.	% infection
Seedling boxes	0.5	32	28.2	30	10.0
Glasshouse pots	1	28	28.3	32	15.6
Outdoor bed	2	26	26.9	34	8.8
Outdoor bed	3	30	23.3	36	22.2
Outdoor bed	12	34	32.4	32	12.5
Outdoor bed	14	32	34.4	35	20.6

The results show that conidia of A. zinniae can remain viable in the soil for at least 14 months in the absence of a susceptible host crop.

Accordingly it is concluded that overwintering of A. zinniae as loose conidia in the soil is important as a potential source of primary inoculum for the infection of a new season's crop.

The results of methods of survival of A. zinniae considered in this study are summarised as follows:-

Method of Survival.	Survival - in months.	Importance as a source of primary inoculum.
1. Loose conidia on seed	29	High
2. Vegetative mycelium in seed.	36+	High
3. Vegetative mycelium in dried host tissue.	12+	Low
4. Overwintering on alternate hosts	12+	Low
5. Free conidia, low density (outdoors)	1	Nil
6. Free conidia, low density (indoors)	1-4½	Nil
7. Free conidia, high density (outdoors)	6	Nil
8. Free conidia, high density (indoors)	15	Low
9. Mycelium in soil particles	3	Nil
10. Mycelium in soil organic matter	3	Nil
11. Crop debris in soil (outdoors)	2	Nil
12. Crop debris in soil (indoors)	13	Nil in sterilised. soil.
13. Loose conidia in soil (Outdoor plantings.)	15	High

From this summary it can be seen that the most important methods of overwintering of *A. zinniae* and hence the most likely sources of primary inoculum for the infection of a new season's crop are seedborne inoculum and free conidia in the soil.

This is in agreement with the work of Baker & Davis (1950) who consider that primary inoculum arises from infected seed sown in the field and from a "soil carryover".

These survival studies have focussed attention on two major substrates for perpetuation of *A. zinniae* in the absence of a susceptible host crop: the seed

and the soil. Inoculum of the fungus, present as conidia in the soil in outdoor plantings, could conceivably be eliminated as a source of primary inoculum by use of a suitable rotation or by soil sterilisation.

Seedborne inoculum on the other hand, because it is not eliminated by storage of the seed for at least three years, can only be controlled by the use of an appropriate seed treatment designed to eradicate the pathogen and yetnot severely affect seed germination capacity.

9. SOME OTHER FACTORS WHICH MAY INFLUENCE THE DISEASE CYCLE.

(a) PREDISPOSITION.

The age of plants and the conditions under which they grow may affect their susceptibility to disease. This environmentally conditioned susceptibility is termed predisposition. According to Yarwood (1959) predisposition is 'the tendenc of nongenetic conditions, acting before infection, to affect the susceptibility of plants to disease.'

Environment affects disease through its direct effect on the pathogen, through its effect on the susceptibility of the host and through its effect on the interaction of host and pathogen. The first category is excluded from predisposition by definition. The second category is the subject matter of predisposition, and the third is sometimes difficult to separate from the second. (Yarwood loc. cit.)

Two factors, plant age and wilting effects, were considered to determine their effects in predisposing zinnia plants to infection by A. zinniae.

Yarwood (loc. cit.) states that -

> 'Experimentally, predisposition can be clearly demonstrated only by exposing otherwise similar healthy plants to contrasting conditions, then placing them all in the same environment, and inoculating them or subjecting them to a disease-inducing environment. If the experimental preinoculation treatments cause differences in disease, predisposition may be said to have occurred'.

(i) Plant Age Predisposition.

Plants were inoculated at 1, 2, 4, 6, 8, 10 and 16 weeks from sowing. Groups of 3 plants were used at each age. All inoculated plants were susceptible to attack, leaf tissue being especially susceptible. Stem attack was often more marked in juvenile plants up to 4 weeks of age. Adult plants (over 8 weeks old) showed some reduced stem susceptibility to attack by A. zinniae, but floral infection increased with flower age.

Zinnia plants were susceptible to infection by the pathogen at all stages

of growth from the seedling stage through to full maturity. If any generalisations can be made they would be that stems of plants appeared to be predisposed to increased susceptibility as seedlings and to decreased susceptibility with maturity, and that floral infection appeared to become more severe with increasing flower age.

Overall, however, it seems that age is not a major factor affecting the predisposition of zinnia plants to increased or decreased susceptibility to attack by A. zinniae.

(ii.) Wilting Predisposition.

Changes in cell turgor are related to host vigour. Reduced turgor could result from reduced water content of tissues, from high osmotic pressure of external solutions, from prior infection, or from other causes (Yarwood 1959).

Permanent wilting is the term used to describe the wilting of plants under conditions when water in the soil is not available for normal plant functions (Subramanian & Saraswathi-Devi 1959).

Zinnia plants in a state of permanent wilt were inoculated with a spore suspension of A. zinniae (16,000 spores per ml.). Fully turgid plants of the same age were also inoculated with the same spore suspension as controls. All plants were placed in a moist atmosphere for 48 hours after which the wilted plants were supplied with water. Disease ratings taken 7 days after inoculation showed that the plants inoculated in a state of permanent wilt were much more heavily infected by the pathogen than the controls. Ten days after inoculation the test plants had completely collapsed due to the disease. Control plants on the other hand, while extensively diseased were still alive.

From this experiment it would appear that the major effect of wilting is to increase the susceptibility of zinnia tissue to attack by the pathogen.

(b) INOCULUM POTENTIAL.

Inoculation experiments with some diseases have shown that the percentage of

visibly diseased plants declines with decreasing concentration of viable units in the inoculum. When the concentration of units falls below a certain critical level none of the inoculated plants may subsequently develop disease symptoms.

Garrett (1959) defines inoculum potential as the energy of growth of a pathogen available for infection of a host at the surface of the host organ to be infected.

Experiments were conducted to determine the critical level of inoculum potential for A. zinniae and to determine the rapidity and extent of disease manifestation at different inoculum levels.

Materials and Methods.

A spore suspension containing 1 x 10^6 spores per ml. was serially diluted to obtain concentrations of spores down to 1 spore per ml.

Uniform sets of 10 zinnia plants were then inoculated with the original suspension and with each of the dilutions made from it.

Disease rating was recorded 10 days after inoculation.

Results and Discussion.

Plants inoculated at the higher levels (1 x 10^6 down to 1 x 10^4) were the first to produce disease symptoms. The level of inoculum load appeared to influence the length of the Disease Cycle, as evidenced by earlier manifestation and a higher number of lesions produced on plants inoculated with spore suspensions ranging from concentrations of 1 x 10^4 to 1 x 10^6 spores/ml. than with lower inoculum levels.

Relative disease incidence was recorded on a scale 0 to 4 and the results are presented in Table 29.

TABLE 29. INOCULUM POTENTIAL and its EFFECTS on DISEASE MANIFESTATION.

Conc. spores per ml.	Expt. 1. Disease rating.	Expt. 2. Disease rating.
1×10^6	4	4
1×10^5	4	3
1×10^4	3	4
1×10^3	1	2
1×10^2	0	1
1×10^1	0	0
1×10^0	0	0

4 = Very Heavy. 3 = Heavy. 2 = Moderate.
1 = Low. 0 = No disease manifestation.

No infection was obtained in one experiment with inoculum concentrations of 10^2 spores per ml. or lower. In the other experiment 4 lesions were found on the plants inoculated with a concentration of 10^2 spores per ml. after 10 days.

At the maximum concentration of 10^6 spores per ml. 97.5% of the leaves of inoculated plants developed at least one lesion.

Several workers have found that inoculum level influences the rapidity and severity of disease development but that the actual level of inoculum capable of causing infection is influenced by the particular environmental conditions during the test period (Cole and Couch 1958, Garrett 1959, Gooding & Lucas 1959). This was also demonstrated in the present study in that variation in the prevailing conditions during each of the two experiments caused a marked variation not only in disease severity but also in the relative level of inoculum capable of causing infection.

Conclusion.

This experiment demonstrates the effect of the level of inoculum on the speed and severity of disease development. Inoculum concentrations as low as 100 spores per ml. were found to be sufficient for disease infection, thus indicating that even relatively low inoculum levels are capable of initiating a further cycle of the disease.

(c) VARIETAL SUSCEPTIBILITY.

Although no detailed experimentation was carried out to determine whether some varieties of Zinnia elegans were more susceptible or resistant to attack by A. zinniae than others, some general observations were made during this study.

It does not appear that any one variety shows any marked resistance to attack by the pathogen.

Some growers are of the opinion that the variety Lilliput is more resistant to leafspot than are the larger flowered varieties. In the present study no consistent evidence was observed to support this.

All zinnia varieties were susceptible to attack by A. zinniae both in the seedling and adult phases of growth. If any generalization can be made, it is that the larger flowered forms of Zinnia elegans such as Giant Dahlia, Giant Cactus and Californian Giants may be more susceptible to blossom infection than the smaller flowered varieties such as Lilliput, Thumbellina and Linearis. Whether this is a result of smaller floral area, to reduced total leaf surface for infection, or to varietal variation in floral susceptibility was not determined.

C. HOST RANGE STUDIES.

Although Alternaria zinniae has a wide recorded host range, few workers have conducted formal studies aimed specifically at determining the extent to which the pathogen can infect unrelated plant species. Accordingly, in the present study, considerable attention was devoted to this topic.

Neergaard (1945) conducted infection experiments on disease free seedlings of 22 species raised aseptically in test tubes, using an inoculum agar squares of approximately 0.5 cm from actively growing colonies. He observed that the fungus "seems to have a preference for the Compositae," and listed the following hosts as being susceptible:-

Brassica oleracea L	Mammuth orangerosa L
Callistephis chinensis Nees.	Nicotina affinis T. Moore.

Capiscum globatum L.	Nicotinia sanderae Hort.
Daucus carota L.	Senecio cruentus DC.
Godetia grandiflora Lindl.	Solanum melongena L.
Lactuca sativa L.	Triticum aestivum L.
Lycopersicum esculentum Mill.	Zinnia elegans Jacq.

In the same account Neergaard also recorded that in the course of germination tests of commercial seedlines, A. zinniae was found causing "spontaneous attacks" on seeds of the following species:-

Callistephis chinenis Nees.	Helianthus debilis Nutt.
Chrysanthemum carinatum Schousb.	Papaver alpinum L.
Cosmos bipinnatus Cav.	

It would appear that in some cases Neergaard failed to complete the requirements for Koch's Postulates normally recognised as being concluaive proof of pathogenicity. There appears to be no mention of reisolation of the pathogen from his infection experiments conducted on seedlings raised aseptically in test tubes, nor in many cases of initial isolation to agar from seed observed in the course of germination tests to be infected with A. zinniae. The only hosts from which Neergaard states that he obtained isolates of the pathogen were from seeds of the following species:-

Callistephis chinensis Nees. (Hungary)

Chrysanthemum carinatum Schousb. (Denmark)

Chrysanthemum sp. (Italy)

Cosmos bipinnatus Cav. (Denmark)

Papaver alpinum L (Holland)

Senecio cruentus L. (Denmark)

Zinnia elegans Jacq. (3 isolates; Holland, Italy, Hungary.)

Consequently it would appear that Neergaard based his determination of the taxonomic position of the fungus pathogenic to different hosts on the basis of symptom expression and, more important, on the morphology and dimensions of spores produced from inoculated plant tissue. To add further doubt as to whether

the fungus observed by Neergaard on different hosts was in fact A. zinniae, no mention is made of the age of the conidia measured, or of the environmental conditions during their development.

Since his original publication in 1945 Neergaard has recorded A. zinniae as attacking the following host plants:-

Centaurea cyanus L. (1947)

Gentiana acaulis L. (1949)

Gerbera jamisonii Hook. (1947)

Impatiens holstia Engler & Warb. (1946)

Reseda odorata L. (1947)

Tagetes erecta L. (1946)

The host range of A. zinniae has since been enlarged as follows:-

Ageratum conyzoides L (Agarwal & Bhave 1959)

Helianthus annuus L. (Hattrass 1948, McDonald & Martens 1963)

Impatiens sp. (Harrison 1959)

Materials and Methods.

In the present investigation studies relative to the host range of A. zinniae were carried out in two ways:

I. Artificial inoculation.

II. Natural field infection.

I. Artificial Inoculation.

Host range studies were conducted on seedlings and/or adult plants. Seedlings were raised in seedboxes and later transplanted to 6" clay pots, 3 plants pe pot. The age of inoculated plants varied with the growth rate of the species under study. Adult plants of the faster growing annual species could be obtained within four weeks of sowing, but some of the perennial species, especially if grown from cuttings or tubers, required up to eight weeks to obtain plants suitable for inoculation. Plants were kept soft and succulent in an endeavour to predispose them to infection.

A range of species were inoculated under glasshouse conditions with a spore suspension prepared from 10 day old cultures obtained initially from naturally infected adult zinnia plants. Sporulation of cultures was induced by ultraviolet light treatment. In each case inoculum concentration was determined using a haemocytometer and varied with the sporulating capacity of the isolate used. The concentration of conidia ranged from 10,000 to 136,000 spores per ml.

In each inoculation experiment a minimum of three plants of the test species were inoculated and a further two plants used as controls. Two potted zinnia plants were also inoculated during each test to eliminate the possibility of a negative result being caused by the inoculum not being viable, or the environmental conditions at, or subsequent to inoculation not being conducive to infection and disease development. Inoculation of zinnia plants was also important in determining whether or not the inoculum used in each test was pathogenic to zinnia.

Two methods of inoculation were used. In the majority of cases small drops of the spore suspension were placed on test plants using s fine nozzled pipette (Method 1). Otherwise a Windex sprayer was used to mist plants tc runoff point with the spore suspension (Method 2). Some of the plants species inoculated had a waxy or shiny cuticle which tended to repel water. In such cases a wetting agent * brushed onto the leaves prior to inoculation was found to aid adherance of spores to the leaf surface.

At no stage in these studies was plant tissue wounded before inoculation. Care was taken to ensure that the nozzle of the pipette did not touch or scratch the tissue surface during inoculation by Method 1.

A special procedure was adopted for the inoculation of Brassica oleracea L. As far as possible without injury, the outermost 2 - 4 leaves of the head were pulled aside and the inoculum placed on the inner leaves which had previously been covered by several layers of leaves. After inoculation the outside leaves were carefully replaced so that the inoculum was located in a moisture saturated

+ Nipasol M. 0.5 gm/pint of sterile distilled water.

atmosphere and adequately protected against drying.

Following inoculation, plants were placed for 48 hours in a cabinet where the humidity was maintained at approximately 100%, and then removed to the glass-house bench. Ten days after inoculation the percentage infection and symptom type were recorded, and reisolations made to PDA. At the same time a disease rating was made, based on the percentage of leaves infected from the total number of leaves inoculated in each test species, a comparative scale 0 to 10 being used. Reisolation of the fungus from lesions present on infected test plants was made by single spore isolation to PDA, and the colony diameter recorded after 10 days incubation at 28°C. Cultural characters of the isolate obtained from inoculated test plants were compared with those of the original isolates of A. zinniae used for inoculation. Conidia from diseased tissue of each plant held at 28°C for 48 hours were placed in a drop of Shear's mounting fluid on a glass slide and the rim of the coverslip sealed with clear nail varnish to make a permanent slide. Conidia were microscopically measured using a stage micrometer. The range of conidial dimensions of thirty spores per isolate were recorded. Range in spore body length was used to compare isolates since the beak length of spores of A. zinniae varies greatly even within the same isolate.

The isolate obtained from test plants was induced to sporulate by the ultraviolet light method and the resultant spore suspension inoculated to two zinnia plants, the total number of leaves per plant ranging from eight to twelve. Ten days after inoculation the disease rating was recorded and the fungus reisolated toPDA. Colony diameter was recorded after 10 days incubation at 28°C, and spore dimensions measured under the microscope.

Thus for each test species the capacity of A. zinniae to produce disease symptoms on different hosts was proved by -

1. Fulfilment of the requirements of Koch's Postulates.
2. Comparison of colony growth of isolates from inoculated test plants and growth rate of reisolations from inoculated zinnia plants.
3. Comparison of the range in spore body length of isolates from host plants and also from zinnia plants inoculated with the isolate recovered from host plants.

Symptom expression of A. zinniae on different host species is illustrated in Plate 15 and 16 (Page 172) and in Appendix V.

II. Natural Field Infection.

Throughout this study isolations were made from leaves of plant species observed in the field to exhibit lesioning superficially similar to those expressed by zinnia plants naturally infected with A. zinniae. Plant species in this group were:-

Ageratum conyzoides L.	Hydrangea opuloides Koch.
Calendula officinalis L.	Senecio cruentus L.
Callistephis chinensis Nees.	Solanum nigrum L.
Helianthus annuus L.	Tagetes erecta L.

Diseased tissue from the above species was subjected to high humidity at 28°C for 36 hours and isolation to PDA made by single spores. Colony diameter was recorded after 10 days growth at 28°C. Spores from naturally infected plant tissue held at 28°C for 48 hours were mounted on permanent slides and conidial dimensions recorded using a microscope stage micrometer, 30 spores being measured per isolate.

Sporulation of the isolates from naturally infected plant species was induced using ultraviolet light and the resultant spore suspension inoculated to (a) the same plant species, and (b) zinnia plants, under glasshouse conditions.

Disease rating and symptom type were recorded 10 days after inoculation. Reisolation from the inoculated plant species and from zinnia leaves was carried out. The resultant isolates were compared regarding cultural characters, growth rate and colony diameter after 10 days incubation at 28°C. Range of conidial dimensions of the isolate from inoculated plant species and from inoculated zinnia plants were also compared.

Results and Discussion.

I. Artificial Inoculation.

The results of host range inoculation experiments are presented in Table 30.

TABLE 30. ARTIFICIAL INCOULATION of DIFFERENT PLANT SPECIES in HOST RANGE STUDIES.

Common name.	Botanical name	Record of Pathogenicity.	Method of inoc.	Plant part.	Inoc. Conc.	Disease rating.	Reisolation to Zinnia. Inoc. Conc.	Reisolation to Zinnia. Disease rating.
Ageratum	A. conyzoides L.	Agarwal & Bhave 1959	1	AL.B	10	2	46	9
				AL.	14	5	32	10
Aster (china)	Callistephis chinensis Nees.	Neergaard 1945	2	AL.B	46	6	32	10
Bean (mung)	Phaseolus aureus Roxbg.	-	1	SC	136	7	60	10
Black Night-shade.	Solanum nigrum L.	-	2	AL	36	6	48	9
Cabbage	Brassica oleracea L.	Neergaard 1945	1	S	90W	1	36	9
Calendula	C. officinalis L.	-	1	AL.B.	52	7	38	10
				AL.B.	66	5	56	10
Carrot	Daucus carota L.	Neergaard 1945	1	AL.	36W	2	82	10
Carrot (wild)	Daucus carota L.	-	1	AL.	44W	1	76	10
Carnation	Dianthus caryophyllus L.	-	1	AL.	66W	2	74	10
Chrysanthemum	C. indicum L.	-	1	AL.	36	5	114	10
Chrysanthemum (annual)	C. carinatum Schousb.	Neergaard 1945	1	AL.S	52	5	14	8
				AL.	14	4	38	10
Cineraria	Senecio cruentus L.	Neergaard 1945 Schreier 1952	2	AL	52	5	34	9
Cornflower	Centaurea cyanus L.	Neergaard 1948	2	AL	14	5	16	10

TABLE 30 (Ctd.)

Common name.	Botanical Name.	Record of Pathogenicity	Method of Inoc.	Plant part.	Inoc. Conc.	Disease rating.	Reisolation to Zinnia. Inoc. Conc.	Reisolation to Zinnia. Disease rating.
Cosmos	C. bipinnatus Cav.	Neergaard 1945	1	AL	130	4	109	10
Cowgrass	Trifolium pratense L.	-	1	AL	44W	3	52	9
Cucumber	Cucumis sativus L.	-	1	AL.S	52	7	80	10
Dianthus	D. plumarius L.	-	1	AL	66W	0	-	-
Godetia	G. grandiflora Lindl	Neergaard 1945	1	AL	86	3	46	10
			1	AL	14	3	36	10
Gypsophila	G. paniculata L.	-	1	AL	36W	2	168	7
							86	10
Hydrangea	H.opuloides Koch	-	1	AL	52	8	54	10
			1	AL	36	3	46	10
Lettuce	Lactuca Sativa L.	Neergaard 1945 Baker & Davis 1952	1	AL	14	5	50	10
Linaria	L. purpurea L (Mill)	-	1	AL	56	3	24	10
Lobelia	L. erinus L.	-	1	AL	28	0	-	-
Lucerne	Medicago sativa L	-	1	AL	44W	1	38	10
Lupin (Russell)	Lupinus polyphyllus Lindl	-	2	AL	90W	5	44	10
Marigold (African)	Tagetes erecta L	Edwards 1957 Neergaard 1956 De Tempe 1956-7	1	AL	32	6	32	9
Marigold (French)	Tagetes patula L	-	1	AL	56	8	44	10
Microlaena	Microlaena sp.R.Br.	-	1	AL	56	0	-	-

TABLE 30 (Ctd.)

Common name	Botanical Name.	Record of Pathogenicity	Method of Inoc.	Plant part.	Inoc. Conc.	Disease rating.	Reisolation to Zinnia. Inoc. Conc.	Disease Rating.
Mignonette	Reseda odorata L	Neergaard 1948 on seeds	1	AL.B	36	3	76	10
Onion	Allium cepa L	-	1	AL	48W	1	36	9
				AL	68W	0	-	-
				AL	35W	0	-	-
Pea (garden)	Pisum sativum L	-	2	AL	90W	5	42	9
Pea (everlasting)	Lathyrus latifolius L	-	1	AL	44W	1	44	10
Pea (sweet)	Lathyrus odoratus L	-	1	AL	44W	2	84	10
Phlox	P. drummondii Hook	-	1	AL	66	0	-	-
Poppy (Iceland)	Papaver nudicaule L	-	1	AL	56	4	38	10
Potato	Solanum tuberosum L	-	1	AL	44	0	-	-
Subterranean clover	Trifolium subterraneum	-	1	AL	130	2	56	9
Suckling Clover	Trifolium dubium Sibth	-	1	AL	130W	2	48	10
Sunflower	Helianthus annuus L	McDonald & Mertens 1963	2	AL	52	7	32	10
				SC	46	8	32	10
Tobacco	Nicotinia tabacum L.		2	AL	36	8	64	10
Tobacco	Nicotinia rustica L		1	AL	66	3	26	9
Tomato	Lycopersicon esculentum Mill	Neergaard 1945	1	AL	14	5	28	10
				AL	16	5	40	10

TABLE 30 (Ctd.)

Common name.	Botanical name.	Record of Pathogenicity	Method of inoc.	Plant part.	Inoc. conc.	Disease rating.	Reisolation to Zinni: Inoc conc.	Reisolation to Zinni: Disease rating.
Wheat	Triticum aestivum L	Neergaard 1945	1	AL	90W	1	60	10
				E	36W	1	17	8
White clover	Trifolium repens L		2	AL	46W	2	63	10
			1	AL	14W	3	10	8
			1	AL	32W	5	60	9
Zinnia (Lilliput)	Z. elegans Jacq.	Pape 1943	1	AL	64	10	18	10
		Neergaard 1945		AL	52	10	4	10
Zinnia (Giant Dahlia)	Z. elegans Jacq.	Pape 1943		AL	14	9	24	10
		Neergaard 1945	2	AL	54	10	26	10
Zinnia (Old Mexico)	Z. haageana Regel		1	AL	45	9	27	10
				AL	52	7	41	10
Zinnia	Z. pauciflora L		1	SC	36	8	52	10
Zinnia (creeping)	Sanvitalia procumbens Lam.		1	AL	53	6	64	10

KEY:

Disease Rating.

0	=	0% infection	6	=	50 - 59% infection
1	=	1 - 9% infection	7	=	60 - 69% "
2	=	10 - 19% "	8	=	70 - 79% "
3	=	20 - 29% "	9	=	80 - 89% "
4	=	30 - 39% "	10	=	90 - 100% "
5	=	40 - 49% "			

W = Wetting agent used.
AL = Adult plant leaf.
S = Seedling
B = Blossom
E = Ear or spikelet.

Inoc. conc. = Inoculation concentration in 1,000's. per ml.

% infection is based on the % of total leaves showing lesioning at 10 days. One lesion per leaf being considered sufficient to regard that particular leaf as being diseased.

The disease rating recorded for each species often showed variation, dependent on inoculum concentration and temperature and humidity conditions during each test. In all cases the isolate from the plant species inoculated with A. zinniae proved extremely pathogenic to Zinnia elegans, disease rating on zinnia ranging from 7 to 10.

Symptom type is recorded in Table 31. Lesion colour ranged from black to brown. The presence of concentric circles in the lesion area due to mesophyll collapse was not evident in all cases, but with some species (e.g. carrot, cosmos, wheat) leaf shape was such that large circular lesions could not develop. It was observed that often only the larger circular lesions showed the pronounced "target spot" effect so characteristic of some Alternaria diseases.

Lesion shape, size and outline were variable. The presence of leaf abnormali due to malformation or colour changes was most marked on plants of Ageratum conyzoides L., Reseda odorata L., Dianthus caryophyllus L., Pisum sativum L., and Nicotina spp.

TABLE 31. DESCRIPTION of SYMPTOM TYPE on ARTIFICIALLY INOCULATED PLANTS.

Plant sp. inoc. with A.zinniae.	Lesion colour.	Max. diam lesions at 10 days. (in cm.)	Outline.	Leaf yellowing.	Concentric ringing.	Leaf puckering.	Lesion shape.
Ageratum	B.	1.5	E	+	+	+	R
Aster	G.B.	1.5	E	+	+	-	R
Bean	G.B.	1.0	E	-	-	-	R
Black Nightshade	B.Br	1.5	E	+	+	-	I
Cabbage	G.	0.5	E	+ M	+	-	R
Calendula	LBr.	1.0	E	+	+	-	I
Carrot	B.Br.	0.4	D	+	-	-	I
Carrot (wild)	B.Br.	1.0	E	-	-	-	R
Carnation	B.Br.	0.4	E	+	-	+	R
Chrysanth.(perr.)	B.	0.9	E	-	+	+	I
Chrysanth.(ann.)	G.Br.	0.8	E	-	N.R.	-	I
Cineraria	G.B.	0.7	E	-	+	-	R
Cornflower	LG.	1.4	E	-	+	-	R
Cosmos	BrB.	1.0	D	-	-	+	I
Cowgrass	G.Br	1.5	E	-	-	+	I
Cucumber	G.B.	0.5	E	+M	+	-	I
Godetia	LG	1.0	E	+	-	-	I
Gypsophila	G.Br.	0.4	E	+	+	-	R
Hydrangea	Br.P	2.0	D	-	+	+	I
Lettuce	LBr.	1.0	E	+	+	+	I
Linaria	B	0.1	E	+	-	-	R
Lucerne	G.Br	0.3	E	+	-	-	R
Lupin	Br	1.0	E	-	+	-	R&I
Marigold (African)	B	1.5	E	+	+	-	R&I
Marigold (French)	B	0.8	D	-	-	-	I
Mignonette	G.B.	1.2	E	+M	-	+	I

(Continued next page.)

TABLE 31 (Ctd.)

Plant sp. inoc. with *A.zinniae*.	Lesion colour.	Max. diam lesions at 10 days. (in cm.)	Outline.	Leaf yellowing.	Concentric ringing.	Leaf puckering.	Lesion shape.
Onion	LG.	3.0	D	-	-	-	I
Pea, garden	G.B.	2.5	E	+M	+	+	I
Pea, everlasting	B.Br	0.1	D	-	-	-	R
Pea, sweet	LBr.	0.5	E	-	-	-	I
Poppy	Br	1.0	E	-	+	-	I&R
Subterranean clover	G.Br	1.8	E	-	-	+	I
Suckling clover	B	0.5	E	+	-	-	I
Sunflower	G.Br	1.5	E	-	+	+	R&I
Tobacco (*N. tabacum*)	B.Br.	2.5	E	+	+	-	R
Tobacco (*N. rustica*)	B.Br.	1.0	E	+	+	-	R
Tomato	G.	0.7	E	-	+	-	I
Wheat	G.	0.6	D	+M	-	-	I
White Clover	G.Br.	1.5	E	-	-	-	I
Zinnia elegans	G.Br.	2.0	E	-	+	-	R&I
Zinnia pauciflora	G.B.	2.0	E	-	+	-	R&I
Zinnia creeping	G.B.	0.9	E	-	+	-	R&I

KEY:

Lesion colour - G. = Grey; Br. = Brown; B. = Black; P. = Purple; L. = Light

Outline - E. = Entire; D. = Diffuse

Lesion shape - R. = Regular; I. = Irregular; I&R = Irregular and Regular.

Leaf yellowing - +M = Presence of leaf yellowing but only around margin of lesion.

+ = Present. - = Absent. N.R. = Not recorded.

With most of the plant species studied, inoculated cotyledons and leaves were susceptible to attack, but the plants, if left on the glasshouse bench following inoculation, often tended to 'grow through' the disease. In some cases newly developed leaves were entirely free from attack. This could indicate that conditions were unsuitable for spore dispersal from infected plant parts to young leaves. Alternatively, environmental conditions in the glasshouse may have been favourable for spore dissemination but unfavourable for expression of the disease on leaves developed after inoculation.

In all cases cultural characteristics of the isolate obtained from inoculated test plants appeared identical with those of the original isolates of A. zinniae used for inoculation.

Comparison of the range in spore body length of isolates from different test plant species and from the test plant isolate reisolated from inoculated zinnia plants are recorded in Table 32. Colony diameter at 10 days (in mm.) of isolates grown on PDA at 28°C are also presented. In most cases the isolate from inoculated test plants and the subsequent isolate from zinnia showed a close similarity in both growth rate and range of conidial dimensions.

TABLE 52. SPORE DIMENSIONS and COLONY DIAMETER of ISOLATES from ARTIFICIALLY INOCULATED PLANTS.

	ISOLATE FROM INOCULATED POTENTIAL HOST SPECIES		ISOLATE FROM HOST SPECIES TO ZINNIA.	
	Colony diam. from PDA - 10 days.	Range of Body-length from 30 spores measured.	Colony diam. from PDA - 10 days.	Range of body-length from 30 spores meas'd.
Ageratum	75	45.1-75.85	80	53.3-79.95
Aster	84	45.1-69.7	85	41.0-63.55
Bean	84	43.05-61.5	82	57.4-88.15
Black Nightshade	80	45.1-69.7	87	53.3-77.9
Cabbage	78	41.0-77.9	82	49.2-77.9
Calendula	80	41.0-63.55	79	57.4-86.1
Carrot	80	36.9-55.35	86	61.5-100.5
Carrot (wild)	81	38.95-75.85	82	36.9-71.75
Carnation	80	49.2-71.75	84	45.1-69.7
Chrysanthemum (perennial)	88	36.9-63.55	88	53.3-77.9
Chrysanthemum (annual)	85	45.1-65.5	84	47.15-71.75
Cineraria	77	38.95-47.15	79	30.75-63.55
Cornflower	80	38.95-75.85	83	32.8-43.05 (10 spores onl
Cosmos	85	51.25-69.7	85	38.95-71.75
Cowgrass	83	36.9-63.55	83	45.1-71.75
Cucumber	81	26.65-59.45	82	47.15-88.15
Godetia	84	49.2-71.75	85	61.5-75.85
Gypsophila	83	36.9-57.5	86	55.35-71.75
Hydrangea	83	38.95-63.55	84	55.35-82.0
Lettuce	81	49.2-71.75	83	49.2-61.75
Linaria	80	49.2-65.6	86	47.15-69.7
Lucerne	82	38.95-77.9	82	45.1-75.85
Lupin	79	61.5-77.9	82	41.0-69.7
Marigold (African)	83	41.0-75.85	84	47.15-75.85
Marigold (French)	85	43.05-69.7	86	51.25-75.85

(Continued next page)

TABLE 32 (Ctd.)

	ISOLATE from INOCULATED POTENTIAL HOST SPECIES.		ISOLATE from HOST SPECIES TO ZINNIA.	
	Colony diam. from PDA - 10 days.	Range of Body-length from 30 spores measured.	Colony diam. from PDA - 10 days.	Range of body-length from 30 spores measured.
Mignonette	86	47.15-77.9	84	61.5-88.15
Onion	75	51.25-67.65	84	32.8-77.9
Pea (Garden)	78	31.25-75.85	80	49.2-82.0
Pea (Everlasting)	80	41.0-67.65	82	36.9-73.8
Pea (Sweet)	79	47.15-71.75	83	45.1-69.7
Poppy	83	51.8-75.85	82	38.95-71.75
Subterranean Clover	83	45.1-65.6	85	51.25-77.9
Suckling Clover	83	53.3-63.55	69+	41.0-75.85
Sunflower	74+	41.0-53.3	82	51.25-75.85
Tobacco(N. tabacum)	82	51.25-71.75	83	55.35-69.45
Tobacco(N. rustica)	74	47.15-63.55	81	49.2-75.85
Tomato	79	36.9-63.55	82	41.0-61.5
Wheat	83	61.5-73.8	83	53.3-88.15
White Clover	79 80	31.25-63.55 41.0-63.55	77 80	41.0-69.7 41.0-71.75
Zinnia (elegans)	86	41.0-65.6	85	45.1-63.55
Zinnia (pauciflora)	80	49.2-69.7	85	69.7-92.25
Zinnia (creeping)	85	51.25-77.9	81	45.1-63.55

+ = Contaminated with bacteria.

Although A. zinniae was proved pathogenic to more than 20 previously recorded host species by artificial inoculation, it is important to note that the majority of these are probably of academic interest only. Because of the mechanism of spread of the fungus by rain splash over short distances, it is unlikely that A. zinniae could establish and incite an important disease on some of these hosts. This is because many of these plant species would not be grown in close association with zinnias (e.g. Allium cepa L., Cucumis sativus L., Daucus carots L, Medicago sativa L, Phaseolus aureus Roxbg.). It is possible that some of the host species recorded, especially cultivated land weed species growing as volunteers in zinnia plantings, could be of some importance as alternate hosts of the fungus in the off-season. Hosts in this category could include Lathyrus latifolius L, Solanum nigrum L, Trifolium dubium Sibth, T. pratense L, T. repens L, T. subterraneum L. This is substantiated in part by the fact that A. zinniae was successfully reisolated from naturally infected leaf lesions on Solanum nigrum plants growing in a diseased zinnia crop.

The conditions of glasshouse inoculation of plant species, using high inoculum concentrations, and the maintenance of high humidity for 48 hours following inoculation, may tend to be more favourable to infection by the fungus than natural conditions of lower inoculum concentration and fluctuating humidity experienced in the field.

II. Natural Field Infection.

Results of natural field infection studies are presented in Tables 33 and 34. The detection of disease symptoms caused by A. zinniae infection under natural conditions is more conclusive proof of the ability of the fungus to infect a particular host than the results of glasshouse inoculation experiments. Natural infection indicates that under suitable environmental conditions and in the presence of viable inoculum certain hosts may show disease symptoms in the field. The only plant species previously unrecorded as hosts of A. zinniae which were found naturally infected in the field were:-

Hydrangea opuloides Koch and Solanum nigrum L.

TABLE 33. COLONY DIAMETER, SPORE DIMENSIONS and DISEASE RATING of ISOLATES OBTAINED from FIELD INFECTION STUDIES.

PLANT SPECIES.	PLANT PART ATTACKED.	DISTRICT OF ORIGIN.	ISOLATION FROM NATUR'L. INFECTED TISSUE.		GLASSHOUSE STUDIES. Inoculation to Host Species.				Inoculation to zinnia plan			
			3.	4.	1.	2.	3.	4.	1.	2.	3.	4.
Ageratum	L	Palmerston N.	79	36.9-65.5	34	2	82	41.0-69.7	24	9	81	31.95-75.85
	L	" "	82	NR	46	4	80	NR	37	10	80	26.65-69.7
Aster	L	Palmerston N.	83	49.2-75.85	28	5	81	55.35-75.85	43	10	82	45.1-69.7
Black Nightshade	L	Palmerston N.	82	45.1-69.7	32	7	80	41.0-71.75	46	10	82	36.9-71.75
Calendula	L	Palmerston N.	81	36.9-75.85	36	6	81	41.0-69.95	36	10	80	47.15-63.55
Cineraria	L	Wellington	79	49.2-65.6	42	4	80	45.1-75.85	38	9	82	41.0-77.9
Hydrangea	L	Lower Hutt	83	36.9-69.7	36	5	80	43.05-71.75	52	10	82	41.0-63.55
Marigold	L	Palmerston N.	83	41.0-71.75	26	7	82	41.0-75.85	24	10	84	49.2-71.75
	B	Wellington	80	NR	18	4	80	NR	20	9	83	NR
Sunflower	L	Palmerston N.	81	31.25-71.75	26	6	78	41.0-63.55	42	10	83	36.9-69.7
Zinnia	L	Auckland	84	36.9-75.85	52	10	85	43.05-65.6				
	L	Christchurch	86	38.95-77.9	66	10	86	36.9-65.6				
	L	Lower Hutt	84	49.2-69.7	18	10	83	41.0-63.55				
	L	Palmerston N.	83	41.0-71.75	83	10	82	45.1-77.9				
	L	Queenstown	78	43.05-77.9	44	10	81	51.25-69.7				
	L	Te Kuiti	83	49.1-69.7	28	9	84	49.2-65.5				
	B	Te Kuiti	82	NR	36	10	84	49.2-71.75				

KEY: 1 = Inoculation concentration; 2 = Disease rating; 3 = Colony diameter. (PDA 10 days 28°C); 4 = Range in spore body length.

L. = Leaf; B = Blossom; NR = Not recorded.

NB. In all cases, cultural characteristics (excluding pigment production) of the isolate from naturally infected plant spp. proved identical with those of the fungus subsequently reisolated from inoculated zinnia plants.

TABLE 34. SYMPTOM DESCRIPTION of A. ZINNIAE ON HOST PLANTS FROM FIELD AND ARTIFICIAL INFECTION.

PLANT SPECIES.	SYMPTOM TYPE ON NATURAL FIELD INFECTED PLANTS.							SYMPTOM TYPE on SAME SPP. INOCULATED UNDER ARTIF.CON						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Ageratum	G.-B.	0.7	E	+	+	+	R&I	B	1.5	E	+	+	+	R
Aster	G.-B.	1.2	E	+	+	-	R	G-B.	2.0	E	+	+	-	R
Black Nightshade	B.	1.3	E	+	+	-	R&I	B	1.8	E	+	+	-	I
Calendula	B.	1.8	E	+M	+	-	I	B-Br.	2.5	E	+	+	-	I
Cineraria	B-G.	2.2	E	+	+	sl.	R	G-B.	1.2	E	-	+	-	R
Hydrangea	B-P.	2.3	D	-	sl.	+	I	B-P.	2.8	D	-	+	+	I
Marigold	B.	0.6	E	+M	+	-	R	B	0.4	E	+M	-	-	R&I
Sunflower	B.	2.7	E	sl.	+	sl.	R.	B.	1.2	E	-M	+	+	R
Zinnia (Auckland)	G-B.	2.2	E	-	+	-	R&I.	G-B.	1.8	E	-	+	-	R&I
(Christchurch)	G-B.	1.6	E	-	+	-	R&I	B	2.2	E	-	+	-	R
(Lower Hutt)	G-B.	1.8	E	-	sl.	-	R	G-B.	2.0	E	-	+	-	R
(Palm. Nth.)	G-B.	2.3	E	-	+	-	R&I	G-B	1.6	E	-	+	-	R&I
(Queenstown)	G-B.	2.2	E	sl.	+	-	R	G-B.	1.9	E	-	+	-	R&I
(Te Kuiti)	G-B.	2.0	E	-	+	-	R	G-B.	0.9	E	-	+	-	R&I

KEY:

1 = Lesion Colour. G = Grey. Br. = Brown
B = Black. P. = Purple.

2. = Maximum lesion diameter in cm.

3. = Outline. E = Entire. D. = Diverse.

4. = Leaf yellowing.

5. = Concentric ringing of lesion area.

6. = Leaf distortion and puckering.

7. = Lesion shape. R = Regular. I = Irregular.
R&I = Presence of both regular and irregular lesions.

\+ = Present. - = Absent.

+M = Present but restricted to margin of lesion only.

All the other naturally infected host species (Table 33) are unrecorded as hosts of A. zinniae in New Zealand (apart from Zinnia elegans), although the pathogenicity of the fungus to some of these species has been reported overseas.

Conclusion.

While it is important to record that A. zinniae was proved pathogenic to 42 out of 47 test plant species inoculated artificially, these results are felt to be of academic interest only. The ability of a fungus to induce natural infection in the field is considered to be more conclusive proof of its pathogenicity than the results of artificial inoculations.

On the basis of

1. Successful reisolation of A. zinniae from lesions produced on host plants under field conditions,

2. Comparison of cultural characteristics of isolates from naturally infected plants,

3. Reisolation of isolates from naturally infected plants inoculated to zinnias.

4. Comparative growth rate measurements of isolates from naturally infected host species and isolates from inoculated zinnias, and

5. Comparison of spore characters and dimensions; the following plant species are concluded to be new hosts for A. zinniae :-

Hydrangea opuloides Koch.

Solanum nigrum L.

On the basis of field infection studies the following plant species, while they have been previously recorded as hosts of the pathogen overseas, are presented as new host records for A. zinniae in New Zealand:-

Ageratum conyzoides L

Callistephis chinensis Nees

Calendula officinalis L

Helianthus annuus L

Senecio cruentus L

Tagetes erecta L

PLATE 15 - Artificially inoculated plants of tobacco (Nicotinia tabacum L.) 10 days after inoculation.

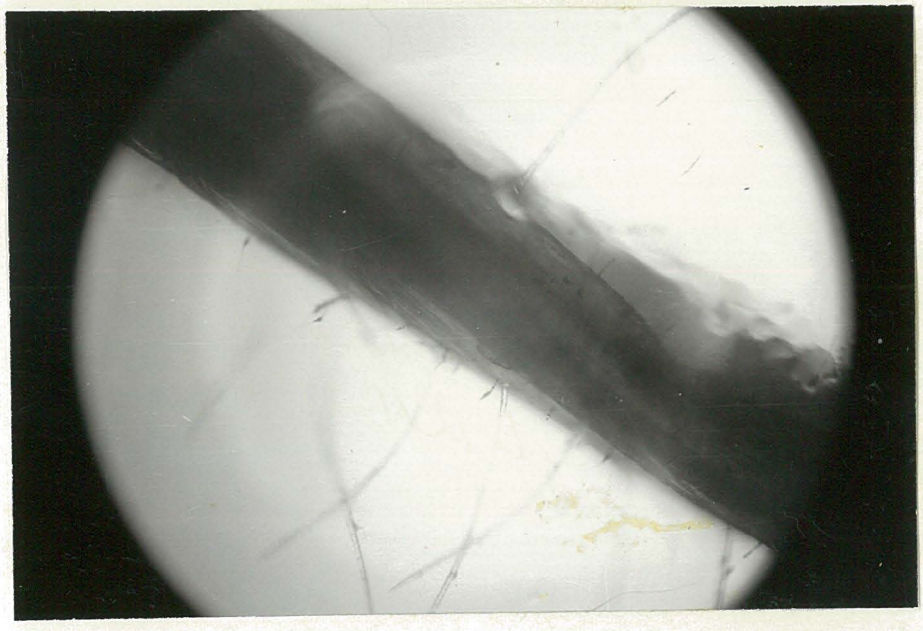


PLATE 16. Conidial production from the stem of an inoculated plant of red Clover (Trifolium pratense L.)

C H A P T E R 3.

DETECTION and CONTROL of ALTERNARIA ZINNIAE ASSOCIATED with ZINNIA SEED.

DETECTION AND CONTROL OF ALTERNARIA ZINNIAE ASSOCIATED WITH ZINNIA SEED.

INTRODUCTION.

Diseases may be seedborne in two ways, namely, (i) the pathogen may be present as free inoculum superficially contaminating the seed surface; and (ii) the pathogen may be established as vegetative mycelium with the testa or embryo tissues of the seed.

Knowledge of the nature of association between the pathogen and seed is basic to any study in which the objectives are the detection of the pathogen in seedlines or disease control by way of seed treatment.

As far as control is concerned, if the pathogen is present only as free surface inoculum, dusting the seed with an appropriate fungicidal dressing would probably be effective in its control. In the case of true seed infection, however, methods of seed treatment to produce internal disinfection of the seed must be employed.

A review of literature on the control of A. zinniae associated with zinnia seed reveals wide variation in the methods used. This is probably due to lack of specific knowledge of the nature of the association between pathogen and seed. Many workers (Neergaard 1945, Dimock & Osborn 1943, Pape 1942 , Forsberg 1946, Baker & Davis 1950, Kispatic 1951, Schmidt 1953, Prota 1960 and Kreitlow 1961) have all reported the pathogen as being seedborne, but few have reported the location of inoculum borne with the seed. Forsberg (1946), Beaumont et al. (1958) Prota (1960) and Kreitlow (1961) state that the fungus is carried on the surface of the seedcoat as external spores. Dimock & Osborn (1943), Pape (1942), Baker & Davis (1950), are the only workers who clearly state the pathogen is borne with the seed as external spores and also as mycelium within the seed. In most other published works, however, the authors have either overlooked or been loathe to make any direct statements on the nature of the association between pathogen and seed; (Kispatic 1951, Schmidt 1953, Edwards 1957, De Tempe 1959 and McDonald & Martens 1963), Most of these workers have readily conceded that the pathogen is seedborne.

This uncertainty as to the type of association between pathogen and seed continues to exist despite the use of chemical and physical methods by many workers to control the pathogen by seed treatment. Some workers have based control methods on the hypothesis that the fungus is borne exclusively on the seed surface, as evidenced by their experiments with surface sterilants. Others have reported the presence of internal as well as external inoculum and accordingly have based control on internal seed disinfection methods.

Because of this confusion it was felt that before any attempts were made at controlling the disease by seed treatment, studies should be undertaken to determine -

a. Whether both contamination and infection occur in zinnia seed imported to New Zealand.

b. Whether, if internal and external inoculum occurs, such inoculum is viable and capable of giving rise to diseased seedlings.

c. The relative importance of the two sites of inoculum.

A. DETECTION OF SEEDBORNE INOCULUM.

I. a. Determination of presence of surface borne spores.

To determine the presence of conidia borne as loose inoculum on the seed surface, 2 grams (approximately 200 seeds) of each of 5 lines of naturally diseased zinnia seed were placed in a 250ml. flask and shaken vigorously with 25ml. of sterile distilled water for 3 minutes. The water was poured from each flask into 4 hard glass tubes which were centrifuged at 1,450 r.p.m. for 10 mins. The supernatent in each tube was discarded and the sludge placed on a slide and examined microscopically for the spores of *A. zinniae*.

Spores of *A. zinniae* were detected in abundance in the seed washing sludge, at a strength of up to 6,000 spores per ml. Conidia were deeply pigmented and in most cases the terminal beak was either absent or broken. Thus it is concluded that conidia of *A. zinniae* may be borne as loose inoculum on the surface of zinnia seed.

C H A P T E R 3.

DETECTION and CONTROL of ALTERNARIA ZINNIAE ASSOCIATED with ZINNIA SEED.

DETECTION AND CONTROL OF ALTERNARIA ZINNIAE ASSOCIATED WITH ZINNIA SEED.

INTRODUCTION.

Diseases may be seedborne in two ways, namely, (i) the pathogen may be present as free inoculum superficially contaminating the seed surface; and (ii) the pathogen may be established as vegetative mycelium with the testa or embryo tissues of the seed.

Knowledge of the nature of association between the pathogen and seed is basic to any study in which the objectives are the detection of the pathogen in seedlines or disease control by way of seed treatment.

As far as control is concerned, if the pathogen is present only as free surface inoculum, dusting the seed with an appropriate fungicidal dressing would probably be effective in its control. In the case of true seed infection, however, methods of seed treatment to produce internal disinfection of the seed must be employed.

A review of literature on the control of A. zinniae associated with zinnia seed reveals wide variation in the methods used. This is probably due to lack of specific knowledge of the nature of the association between pathogen and seed. Many workers (Neergaard 1945, Dimock & Osborn 1943, Pape 1942, Forsberg 1946, Baker & Davis 1950, Kispatic 1951, Schmidt 1953, Prota 1960 and Kreitlow 1961) have all reported the pathogen as being seedborne, but few have reported the location of inoculum borne with the seed. Forsberg (1946), Beaumont et al. (1958) Prota (1960) and Kreitlow (1961) state that the fungus is carried on the surface of the seedcoat as external spores. Dimock & Osborn (1943), Pape (1942), Baker & Davis (1950), are the only workers who clearly state the pathogen is borne with the seed as external spores and also as mycelium within the seed. In most other published works, however, the authors have either overlooked or been loathe to make any direct statements on the nature of the association between pathogen and seed; (Kispatic 1951, Schmidt 1953, Edwards 1957, De Tempe 1959 and McDonald & Martens 1963), Most of these workers have readily conceded that the pathogen is seedborne.

This uncertainty as to the type of association between pathogen and seed continues to exist despite the use of chemical and physical methods by many workers to control the pathogen by seed treatment. Some workers have based control methods on the hypothesis that the fungus is borne exclusively on the seed surface, as evidenced by their experiments with surface sterilants. Others have reported the presence of internal as well as external inoculum and accordingly have based control on internal seed disinfection methods.

Because of this confusion it was felt that before any attempts were made at controlling the disease by seed treatment, studies should be undertaken to determine -

a. Whether both contamination and infection occur in zinnia seed imported to New Zealand.

b. Whether, if internal and external inoculum occurs, such inoculum is viable and capable of giving rise to diseased seedlings.

c. The relative importance of the two sites of inoculum.

A. DETECTION OF SEEDBORNE INOCULUM.

I. a. Determination of presence of surface borne spores.

To determine the presence of conidia borne as loose inoculum on the seed surface, 2 grams (approximately 200 seeds) of each of 5 lines of naturally diseased zinnia seed were placed in a 250ml. flask and shaken vigorously with 25ml. of sterile distilled water for 3 minutes. The water was poured from each flask into 4 hard glass tubes which were centrifuged at 1,450 r.p.m. for 10 mins. The supernatent in each tube was discarded and the sludge placed on a slide and examined microscopically for the spores of *A. zinniae*.

Spores of *A. zinniae* were detected in abundance in the seed washing sludge, at a strength of up to 6,000 spores per ml. Conidia were deeply pigmented and in most cases the terminal beak was either absent or broken. Thus it is concluded that conidia of *A. zinniae* may be borne as loose inoculum on the surface of zinnia seed.

b. Determination of the presence of deepseated inoculum.

To determine whether the pathogen was present within the testa and embryo tissues, seed dissection was carried out. Attempts to remove the embryo from the seedcoat by soaking seed in 5% NaOH for 24 hours and subsequent extraction of the embryo by gently rubbing the seed between the fingers proved successful.

Many of the separated embryos showed discolouration. Lesions present on the radicle and cotyledons of the embryo ranged from black to dark brown in colour and were often slightly shrunken in appearance. Some of these lesions covered the entire embryo surface while in other cases the lesioned area was confined to one or both cotyledons only.

PLATE 17. Lesioned embryos obtained by dissection of naturally diseased zinnia seed.

To determine whether fungal mycelium was present in the lesions on diseased embryos, a tissue clearing procedure similar to that described by Simmonds (1946) was used. The embryos were allowed to remain in an excess of water overnight to leach out most of the NaOH. The water was then drained off and 95% alcohol added. A sample (20) of the lesioned embryos were placed in a watch-glass, the excess alcohol drained off and clove oil added. After 2 hours the embryo lesions were examined for fungal mycelium. Some difficulty was experienced in distinguishing fungal hyphae from seed tissue. Septate mycelium was observed in some of the lesioned areas but details of the association between fungal tissue and the almost transparent host cells could not be determined. This embryo clearing method, however, did allow positive identification of the presence of septate mycelium in the lesioned areas of embryos dissected from ungerminated seed.

II. DETERMINATION OF VIABILITY OF INOCULUM ASSOCIATED WITH SEED.

(a) As External Spores.

A spraying technique was used to determine whether loose conidia on the seedcoat of naturally diseased lines of zinnia seed were in fact viable. A spore suspension was prepared by washing loose spores from the surface of 3 grams of a naturally diseased line of zinnia seed by the method described in Ia. The spore concentration was determined using a haemocytometer (3,200 spores per ml.). The spore suspension was atomised onto the leaves of 10 healthy zinnia plants. Two disease free plants were atomised with sterile distilled water as controls. The plants were held at glasshouse temperature under high humidity for 48 hours and then removed from the high humidity chamber to the glasshouse bench. A count of the number of leaves that developed symptoms was taken at 10 days. One lesion per leaf was considered sufficient to indicate infection. Reisolations from lesioned tissues was made by subjecting leaves to high humidity in the laboratory for 48 hours and transferring single spores to slopes of PDA.

Of the ten plants (76 leaves) inoculated with the seed washing spore suspension, 89.5% (68 leaves) had developed disease symptoms 10 days from inoculation. Control plants were entirely disease free at 10 days. Successful reisolations of the pathogen from lesions on leaves of inoculated plants were identical with cultural characteristics of isolated of A. zinniae from naturally diseased zinnia plants, in all cases.

These results are not surprising when it is considered that the conidia of some imperfect fungi may survive for very long periods. The results of the present experiment concur with those described previously (page 118) where loose conidia of A. zinniae were found to retain their viability on the surface of zinnia seed for up to 30 months from harvest.

From these results it is concluded that the loose spores adhering to the surface of naturally diseased lines of zinnia seed imported into New Zealand are viable and capable of causing the development of disease symptoms when inoculated onto the leaves of disease free plants.

b. As Internal Mycelium.

A plating technique was used to determine whether the lesions shown by seed dissection to be present within the embryo contained viable inoculum. The use of the NaOH soaking method (1.b) to separate the embryos from the seedcoats was successful, but the chemical was unfortunately lethal to the fungus present in embryo tissues. Accordingly, manual dissection of the seed by peeling off the testa with a sharp scalpel was used,although this procedure was much less convenient.

Following dissection the separated embryos and testas were washed in running water for 15 minutes prior to drying between two sheets of sterile blotting paper and plating to prune agar. The plates were incubated at 28°C for 4 days. Fungal colonies arising from plated tissue were cut with a corkborer and the agar

circles examined for spore production and identification after incubation at room temperature for 48 hours.

Results of seed tissue plating are presented in Table 35.

TABLE 35. RECOVERY OF A. ZINNIAE FROM EMBRYOS AND TESTAS OBTAINED FROM DISSECTED SEED AND PLATED TO PRUNE AGAR.

	Number plated.	Number of fungal colonies.	Number yielding A. zinniae.	% A. zinniae
Embryos	192	32	16	8.3
Testas	158	53	14	8.9

In both cases the majority of plated tissue pieces not yielding A. zinniae were either devoid of mycelium or yielded A. tenuis (embryos 2.1%, testas 14.6%) or Penicillium app.

These results prove that true seed infection may occur, the pathogen being successfully established within tissues of both the testa and embryo of the seed.

c. Determination of whether lines carrying surface inoculum only can produce diseased seedlings.

It has previously been confirmed that mycelium present in lesions on the embryos of ungerminated seed are capable of growth when such tissue is plated to agar. It remained to determine whether loose inoculum on the seed was capable of infecting seedling tissue during seed germination, and hence of producing diseased plants.

The seed used was 1963 seedline of variety Giant Dahlia Flowered which on sowing had yielded 0.5% diseased seedlings. Seed from this line was divided into six parts and a random lettering system used to allocate the treatment received by each portion and to eliminate bias in recording results.

Seed was contaminated with spores of A. zinniae in the following ways:-

1. Glass beads (2mm diameter) were moistened and rolled over the surface of cultures of *A. zinniae* on PDA which had been induced to sporulate under ultraviolet light. The beads with their adhering spores were then shaken for 3 minutes with dry seed in a flask.

2. A 5ml. portion of spore suspension (52,000 spores/ml.) was poured onto dry seed in a 250 ml. flask. The seed was shaken for 1 minute and tipped out onto blotters to dry for 48 hours.

3. 50 ml. of spore suspension (52,000 spores/ml) was poured onto dry seed in a 250 ml. flask. The seed was allowed to soak in this suspension for 12 hours and then tipped out onto blotters to dry for 48 hours.

4. A spore suspension (60,000 spores/ml.) was atomised onto dry seed spread out thinly on blotters. The seed was allowed to dry for 48 hours.

5. Slightly moistened seed was shaken with ten heavily sporulating diseased zinnia leaves in a 250 ml. flask for 3 minutes and the seed allowed to dry for 48 hours, on blotting paper.

Untreated seed was used as a control.

The seed in each sample was weighed to allow calculation of the approximate number of seeds present, and sown in boxes of sterilised soil in the glasshouse.

The number of seedlings producing cotyledonary lesions was recorded at 10 days. Lesioned cotyledons were removed to the laboratory and placed under high humidity at 28°C for 48 hours. Reisolations were made from single spores to slopes of PDA.

Percentage germination figures from 200 seeds per treatment were obtained by the folded towel method. Germination counts for untreated seed were also recorded.

The effect of the above treatments in inducing disease in seedlings and on seed germination are presented in Table 36.

TABLE 36. THE ABILITY OF LOOSE SPORES TO CAUSE DISEASED SEEDLINGS, WHEN CONTAMINATED SEED IS SOWN IN SOIL.

Treatment Method.	Seed weight grams.	Approx. no. Seeds sown.	No. lesioned Cotyledons.	% Cotyl. Lesions.	Germination % 3 days	7 days.
Untreated.	4.34	385	2	0.6	76	82
1	7.46	660	22	4.14	77	80
2	6.01	530	51	12.3	71	78
3	7.09	625	54	11.8	68	77
4	4.54	400	21	5.25	74	80
5	6.13	540	25	5.6	70	82

These results show that the percentage of diseased cotyledons arising from seed which had been surface contaminated with conidia is much higher in all treatments than the percentage of cotyledonary lesioning produced from untreated seed.

A completely disease free line could not be used for this experiment since all lines of seed screened (25 lines in 1962, 101 lines in 1963) showed some disease symptom development. However, this line of variety Giant Dahlia Flowered which had yielded 0.5% *A. zinniae* from screening trials, was the lowest diseased seedline available, and it is felt that the higher number of diseased seedlings following treatment could not have arisen from this small amount of inoculum.

From this experiment it is concluded that loose conidia can act as inoculum for the development of the disease on seedlings arising from contaminated seed. Th mechanism of seedling infection possibly involves the germination of spores on the surface of the testa and penetration into the seedling during seed germination and/or the washing of spores adhering to the seedcoat onto the cotyledons before the testa falls to the ground following germination. This would be greatly aided by overhead watering during the first few days of seedling growth. Spore germination on the seedcoat and penetration of mycelium through the testa and

into the cotyledon tissue may also occur.

d. Determination of the Relative Importance of Surface and Deepseated Inoculum Associated with Zinnia Seed.

Results of previous experiments have shown that the fungus is capable of seed carriage (a) on the seed surface, and

(b) within the testa and embryo.

It was felt desirable to determine the relative importance of each method of transference of inoculum with zinnia seed.

This experiment was carried out using 20 zinnia seedlines which on sowing had produced varying percentages of diseased seedlings (8 to 25%) 10 days from sowing. It was reasoned that if each line was first dusted and then sown, any diseased seedlings appearing must have become infected by means of inoculum deep i the seed. By subtracting the number of diseased seedlings in sowings of the duste sample from the number of diseased seedlings in sowings of untreated seed of each line the extent to which seed contamination was present could be determined.

Seed was counted out in lots of 200 (800 seeds per line). An initial 200 seeds were assessed for germination percentage by the folded towel method at 25°C, and a further 200 untreated seeds per line were sown in sterilised soil in the glasshouse.

The treatment used to remove surface borne inoculum from the seed involved dusting the seed with thiram.

A sample of 400 seeds from each line was placed in a 100 ml. beaker and shaken with Fernasan[a] dust for 3 minutes to obtain complete seed coverage. A volume of dust approximately equal to the volume of one seed of the variety being dusted was used. The seed was then tipped onto a 1mm mesh screen and shaken to remove excess dust. Seed was germinated by the folded towel method to assess the effect of the dusting on seed viability and a further 200 dusted seeds from each line sown in sterilised soil.

[a] = An I.C.I. product containing as active ingredient Tetramethyl thiuram disulphide 50%.

In this experiment sowing trials only were carried out to measure the disease percentage because this method is a reflection of the results a grower would obtain.

Seedlings emerged from untreated and dusted samples of each line were examined for cotyledonary lesioning ten days after sowing. The number of diseased cotyledons present was recorded.

To determine whether the fungus was viable in seedling lesions arising from "untreated" and "Dusted" seed, approximately 75 diseased cotyledons from each treatment were removed to the laboratory. These were washed for 4 hours in running tap water and bisected medianally. One half of each cotyledon was plated to prune agar and the other half given high humidity treatment. Plates were incubated at 28°C for 4 days. Fungal colonies arising from plated tissue were cut with a corkborer and the agar circles examined for sporulation and identification after incubation at room temperature for 48 hours. Tissue pieces given high humidity treatment were examined for spore production after 2 days incubation at 28°C.

Results of disease incidence from sowing trials are recorded in Table 37.

TABLE 37. DETERMINATION of the RELATIVE IMPORTANCE of SURFACE and DEEP-SEATED INOCULUM ASSOCIATED WITH ZINNIA SEEDLINGS.

Seedline.	Germination percentage (7 days). Untreated	Dusted.	No.diseased cotyl. (10 days) Untreated	Dusted.	Redtn.in lesions due dusting.	Importance Contamntn. Infection.
Elegans pumila	76	71	16	3	13	13/3
Lilliput pastel mixed	87	85	10	7	3	3/7
Fantasy mixed	73	73	12	7	5	5/7
GDF Luminosa	57	56	9	6	3	3/6
Purple Prince	82	79	16	12	4	4/12
Meteor GDF	74	74	20	0	20	20/0
GDF Crimson monarch	86	78	11	7	4	4/7
Persian Carpet	92	92	12	3	9	9/3
GCF Dark Jewels	88	87	14	9	5	5/9
California Giants	93	88	18	9	9	9/9
Lilliput Golden Gems	88	87	10	6	4	4/6
GDF Golden State	86	80	19	6	13	13/6
GDF Royal Purple	80	77	16	14	2	2/14
Lilliput Black Ruby	86	86	21	5	16	16/5
Persian Carpet	92	92	8	0	8	8/0
GDF Eldorado	85	77	14	4	10	10/4
Lilliput Pompom	82	80	12	0	12	12/0
GDF Finest Mixed	88	87	9	1	8	8/1
Elegans pumila dwf.	59	59	10	4	6	6/4
Super Giants	71	71	24	8	16	16/8

KEY: G.D.F. = Giant Dahlia Flowered.

G.C.F. = Giant Cactus Flowered.

The use of the figures 13/3 to indicate the relative importance of inoculum associated with seedline 1 in Table 37 (Elegans pumila) is interpreted following sowing trials by the following:-

1. Percentage diseased seedlings produced from untreated seed = 13+3 = 16%.
2. Percentage of diseased seedlings produced as a result of surface inoculum = 13%.
3. Percentage of diseased seedlings produced as a result of deep-seated inoculum = 3%.

Thus the relative importance of contamination and infection of this seedline is designated 13/3.

The number of plated lesions, and those lesions given high humidity treatment which yielded A. zinniae are recorded in Table 38.

TABLE 38. RECOVERY OF A. ZINNIAE FROM DISEASED COTYLEDONS ARISING FROM UNTREATED AND DUSTED SEED.

Seed Treatment.	No. cotyl. plated.	No. plated cotyl. yielding A. zinniae.		No. high R.H. cotyl. yielding A. zinniae.	
		No.	%	No.	%
Untreated	73	52	71.2	37	50.7
Dusted	75	44	58.7	18	24.0

By the method described in this experiment it is possible to determine the relative importance of surface and internal inoculum associated with any seedline. The results from the use of this method can yield important information on whether the dusting of seed is likely to be effective as a control method because of the predominance of surface inoculum, or whether internal seed disinfection is necessary to control high levels of deepseated inoculum.

The experiments in this section have shown:

(a) The presence and viability of both surface and internal inoculum associated with zinnia seed.

(b) That conidia borne on the seed surface can cause the production of diseased seedlings.

(c) The use of a method to determine the relative importance of internal and external inoculum of A. zinniae associated with naturally infected seedlines of Zinnia elegans.

B. SEED SCREENING TRIALS.

Before any experiments were carried out to determine the efficiency of different seed treatments in the control of inoculum of A. zinniae associated with zinnia seed, seed screening trials were conducted to develop a rapid reliable and practical method for the detection of the pathogen in seedlines. These studies were required to determine the extent to which the pathogen is being annually introduced with seed of Zinnia elegans imported to New Zealand.

The following methods were investigated to determine their suitability for seed screening studies:-

1. Visual examination of the seed.
2. Centrifuge technique.
3. Germination of seed on blotters.
4. Plating seed to agar media.
5. Ultraviolet light irradiation of seed.
6. Sowing seed in sterilised soil.
7. Embryo examination technique.

1. Visual examination of seed.

Visual examination of zinnia seed often revealed a percentage of the seeds bearing dark brown spots externally. These spots may be up to 1mm. in diameter and occur at random over the seed surface rather than being consistently associated with a particular area of the seedcoat. The spots may extend into the testa although often such a seed produces a normal seedling. To determine whether fungi were associated with these spots, plating trials were carried out.

Surface spots were dissected from the testa of a seedline of variety Giant Dahlia Flowered using a fine scalpel. Tissue pieces were placed in a muslin bag and washed vigorously in running tap water for 6 hours, then dried on sterile blotting paper for 15 minutes and plated to prune agar. Plates were incubated at 28°C for 5 days. Fungal colonies arising from plated tissue were cut out with a 5mm. corkborer and the agar circles placed inside a petri plate lid. After 48 hours incubation at room temperature the rims of the agar circles were examined for spore production.

No one fungus was consistently reisolated from the 100 seed spots plated to agar, cultures of species of Alternaria, Penicillum, Rhizopus and Botrytis were obtained.

The numbers of fungal colonies identified from 100 seed spots plated to culture are recorded below:-

Alternaria zinniae	9
A. tenuis	12
Penicillium spp	5
Rhizopus spp	4
Botrytis spp	1
Others	2
Total -	33

These results are not unlike those obtained by Gloyer (1931), who plated "brown spots on the distal and basal portions of the seed" of Callistephis chinensis Nees to artificial media. Gloyer was also unable to obtain consistent reisolations of one predominant fungus from such spots.

In the present study it was concluded that while visual examination of seed may give some indication of the presence of seedborne pathogens, this criterion is not of great value in determining the presence or absence of A. zinniae associated with zinnia seed.

2. Centrifuge technique.

Initial studies revealed that conidia adhering to the seed were almost impossible to detect consistently by microscopic examination. Accordingly, the centrifuge technique was employed to determine their presence and to gauge approximate spore load on individual seedlines.

The method used was the same as described in Section A. part I (a) of this chapter. The number of spores for a known amount of seed (the spore load) was determined microscopically with a haemocytometer. This technique revealed that in 20 seedlines studied by this method, surface borne inoculum of A. zinniae was present to a greater or lesser extent in all seedlines, with spore loads up to 8,000 per ml.

A modification of the centrifuge technique which reduced the time required in processing seed washings, was also studied. A large quantity of seed could be tested for surface borne spores by this method, which consisted of shaking the seed vigorously in a flask containing sterile distilled water and filtering the resultant solution through filter paper on a Buchner funnel under suction. The spores were collected on the filter paper, which could then be examined microscopically under a 40X binocular microscope for identification of spores and for spore counts per unit area.

The centrifuge method, while useful for screening large numbers of seedlines, provided only a relative estimate of surface borne spores. The method could only be used to grade seedlines into classes of high, moderate or low amounts of seed contamination. The major objection to the use of this method of screening seedlines was that it did not give any indication of internally borne inoculum. The absence of spores as shown by the centrifuge method, while it may indicate the absence of surface borne inoculum, in no way ensures that the particular seedline under study is free from the pathogen. Where spores are detected in seed washings by the centrifuge method there is still no evidence of the viability or pathogenicity of such spores. For this method to be of use in detecting diseased

seedlines in screening experiments it would thus be necessary to supplement the centrifuge technique with a pathogenicity test such as the spraying of seed washi onto healthy zinnia plants and reisolation of the pathogen from subsequent disease lesions. Thus this method was considered impractical for use as a screening meth especially since it only gives an index of seed contamination and further tests would be required to test for the presence of internally borne vegetative myceliu of the pathogen in zinnia seed.

3. Blotter Test.

The method of placing untreated seeds on moist blotters and incubating them at a specified temperature has long been used as a recognised technique in Seed Health Testing. After incubating the seeds for a prescribed period, the pathogens are identified on the basis of disease symptoms on the seedlings or microscopic examination of individual seeds. When the blotter test is used to obtain reproducible results the incubation temperature, time, and intensity of lighting must be standardised.

In the present study zinnia seed was germinated on moist blotters in bakelite trays (200 seeds per line). All trays were held at 25°C in a Copenhagen seed germinator for 10 days under natural diurnal light variation. After 10 days counts were made of the number of seedlings which had developed disease lesions. Where there was some doubt as to the pathogen causing lesioning on a particular seedling, positive identification was made under a binocular microscope.

Germination counts of the number of normal seedlings were made at 3 and 7 days from placing seed on blotters.

The blotter method gave consistently good results, its main disadvantage being the amount of germinator space required for each sample and, as a result, the relatively low numbers of seeds which could conveniently be used for each test

The advantages of the method were that it recorded pathogenicity of the fungus and the results could be interpreted quantitatively.

The results obtained using the blotter test on 20 test samples of zinnia

seed indicated that the method gave disease ratings similar to those observed in glasshouse sowings in sterilised soil.

4. Agar Plating.

The agar plating method is used widely for detection of fungal pathogens. Surface disinfected or untreated seeds are placed on any of a variety of agar media. The pathogens grow from the seeds and are identified by their colony characteristics. Malt agar and PDA are most commonly used. Three plating methods were used in the present study:-

(a) Ulster technique - involving direct plating of seed to 2% malt agar.

(b) Direct plating of seed to prune agar.

(c) Surface sterilisation of seed in 0.1% mercuric chloride for 2 minutes followed by washing seed in sterile distilled water and plating to PDA.

Methods (a) and (b) proved to be very successful except that it was found difficult to distinguish between colonies of A. zinniae and A. tenuis. Consequently the agar circle method was used to give positive identification of colonies arising from plated seed after the plates had been incubated at 28°C for 4 days.

Method (c) was satisfactory, but although colonies of A. zinniae arising from the seed occurred in many cases, it was felt that methods (a) and (b), which did not involve surface sterilisation of seed, gave a more complete picture of the total disease status of a particular seedline.

The agar plating method is of limited use when large numbers of seedlines are being screened for disease incidence since it requires specialised equipment and training. The difficulty of testing a representative sample of a bulk of seed, using the small number of seeds to which this method is limited, may also reduce the usefulness of the method.

5. Ultraviolet Light Irradiation of Seed.

Ultraviolet light irradiation of seed to stimulate sporulation of A. zinniae from the seedcoat, and subsequent microscopic examination for spores was investigated as a screening method.

The ultraviolet source used was a single tube WL50 fluorescent lamp.

Two materials, pyrex glass and cellophane, were used as UV transmission media.

The lids of petri plates which contained 100 seeds per test were lined with moist filter paper to maintain high humidity.

Metal screw bands were covered with cellophane, the latter being secured with transparent adhesive tape. One hundred seeds per test were placed on the cellophane and a petri plate lid lined with moist filter paper placed over the rim of the screw band to maintain high humidity.

The seeds in each type of container were placed on a wire support 30 cm. from the UV lamp and irradiated through the pyrex or cellophane bases.

For each UV transmission medium the following variables were considered to ascertain their effect on promotion of sporulation of A. zinniae from the seed.

(a) Pretreatment of seed:
- (i) No soaking.
- (ii) Soaking for 12 hours in tap water at room temperature.
- (iii) Soaking for 24 hours in tap water at room temperature.

(b) Duration of UV irradiation for periods ranging from 1 to 48 hours.

(c) Light and dark during the 48 hours incubation period following irradiation.

A mazda Reflector 150W Floodlight 30 lamp provided light and also held the temperature of the plates at 25-28°C throughout the incubation period. For treatments requiring dark folowing irradiation the plates were wrapped in aluminium foil and held under the same conditions as plates subjected to light during incubation.

The seed used was from a line of variety Giant Dahlia Flowered which had developed approximately 15% diseased seedlings from sowing trials in the glasshouse.

The results of this trial are shown in Table 39, figures representing the percentage infection as recorded by microscopic examination of 100 seeds per test.

TABLE 39. THE EFFECT of ULTRAVIOLAT LIGHT in STIMULATING SPORULATION OF *A. ZINNIAE* from the SEEDCOAT of DISEASED ZINNIA SEED.

Presoak hours.		Duration exposed to Ultraviolat Light - (hours) 0		1		3		6		12		24		36		48	
		L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
0	PD	0	0	0	0	0	0	0	0	1	0	-	-	-	-	1	0
0	C	0	0	0	0	0	0	-	0	-	-	3	-	-	-	0	0
12	PD	1	0	5	1	12	4	14	10	8	5	5	4	4	-	3	5
12	C	0	0	5	2	10	2	14	7	6	2	3	3	1	-	2	3
24	PD	1	0	0	2	5	6	7	5	7	3	8	7	5	2	10	5
24	C	1	1	0	0	5	1	7	0	6	1	3	3	5	4	8	8

KEY: PD = UV transmission through bottom of pyrex petri plate.

C = UV transmission through cellophane.

L = Light during incubation period.

D = Dark during incubation period.

\- = Not recorded.

The results in Table 39 indicate that:

1. The exposure of seed to UV light could be used as a screening method for lines of zinnia seed imported to New Zealand.

2. The procedure for optimum expression of the pathogen as shown by spore production from the seedcoat consisted of (i) Presoaking seed for 12 hours, (ii) UV irradiation for 3-6 hours, and (iii) Incubation of seed under continuous light for 48 hours.

3. Soaking of seed appeared to be an essential prerequisite to irradiation.

4. Long durations of UV exposure of seed (up to 48 hours) appeared to have no greater stimulatory effect on sporulation than shorter periods of 3 - 6 hours.

5. In many cases the pyrex petri plate gave superior results to the use of cellophane as a transmission medium for UV irradiation.

6. The minimum period for maximum expression of sporulation from the seed would be approximately 66 hours; i.e. 12 hours soaking, 6 hours UV irradiation and 48 hours incubation.

Although the use of UV irradiation of seed is effective where specialised equipment is available, it is inclined to prove tedious where large numbers of seedlines are to be screened. Its use may be warranted when a quick result on the disease incidence of A. zinniae associated with a small number of seedlines is required.

6. Growing Seeds in Sterile Soil.

By sowing seed in sterilised soil it is possible to record the incidence of seedborne disease associated with seedlines by a direct count of the number of seedlings which develop symptoms. The procedure found to be most suitable in these studies was essentially the same as the indexing method described by Baker & Davis (1950).

Seed was sown thickly in sterilised soil (at least 200 seeds per 8" x 6" seedbox) and covered thinly with sterilised soil. The soil in each box was kept very wet for 10 days in the glasshouse by holding the boxes in trays of water. If the pathogen was present it was manifest by cotyledonary lesioning on seedlings and to a lesser extent by lesions girdling the stems at or above soil level The disease rating or incidence for a particular seedline was recorded by counts of the number of diseased seedlings. This gave a reliable method for determining the amount of disease in particular seedlines, but invariably gave an exaggerated figure unless data was taken before secondary infection occurred (usually 14-16 days from sowing).

7. Embryo Examination.

The embryo method was developed for determining the presence of loose smut of barley and wheat (Simmonds 1946, Russell 1950, Russell & Popp 1951).

Seeds are softened with sodium hydroxide to remove the embryos, which are then cleared in chloral hydrate or lactophenol,and examined microscopically

for the presence of fungal hyphae. The hyphae are more clearly distinguished from seed tissue by staining with cotton blue (Anderson & Leach 1961).

The embryo examination method as described in Section A, part I (b) of this chapter was found to be extremely time consuming and tedious when used as a seed screening technique. Cleared embryos, when examined microscopically, often showed the presence of septate mycelium in some lesioned areas, but this examination gave no positive identification of the fungus concerned. Hence the use of this method was not considered suitable for screening tests on zinnia seedlines.

Summary of technique studied for suitability as methods of screening seedlines of Zinnia elegans for A. zinniae.

	Advantages.	Disadvantages.
1. Visual examination of seed.	Speed.	Results unreliable.
2. Centrifuge technique	Speed. Allowd large amount of seed to be screened in eaoh test.	No indication of fungal viability or pathogenicity . Detects contamination only.
3. Blotter test.	Detects presence, viability and pathogenicity	Time and space consuming. Representative seed sampling problem.
4. Agar plating.	Detects presence, and viability	No indication of pathogenicity Problem of representative seed sampling. Requires special equipment and technical competenc
5. Ultraviolet irradiation of seed.	Speed. Detects presence of pathogen.	No indication of viability or pathogenicity. Microscopt examination time consuming.
6. Sowing seed in sterile soil	Results close to field performance. Detects presence, viability and pathogenicity.	Time and space consuming.
7. Embryo method.	None	Time consuming. Problem of representative seed sampling. No indication of viability or pathogenicity.

From a study of the methods as outlined above it appeared that the blotter method and sowing seed in sterilised soil, although they required longer for the

completion of a test, gave a more complete result of disease incidence than any of the other methods. Accordingly these two methods were used for screening lines of zinnia seed imported to New Zealand in 1962 and 1963. Both methods were used to test each seedline, results from the blotter method being taken after 7 days an results from the sowing method after 10 days.

In 1962, 25 seedlines were screened for the presence of A. zinniae. In 1963 101 seedlines were tested.

In both years no seedline was found to be completely disease free and the percentage of the disease, as recorded by the number of seedlings which developed symptoms, ranged in 1962 from 0.5 to 21.5%, and in 1963 from 0.5 to 16%. Figures for disease incidence on each seedline tested are recorded in Appendix VI.

The results of seed screening experiments would indicate that, in order to prevent A. zinniae from being introduced with the seed, all zinnia lines imported to New Zealand should be treated with an appropriate control treatment. On this basis further work was carried out to determine the effectiveness of various seed treatments to eradicate A. zinniae carried with zinnia seed.

In overseas literature on the control of A. zinniae associated with zinnia seed it is not uncommon to find workers using seedlines in their experiments which are up to 100% infected with the pathogen. In comparison, in the present screening trials, the highest disease incidence recorded was 21.5%. This could indicate that there is a marked reduction in the amount of viable inoculum on or in the seed between harvest in the country of origin and sowing in New Zealand.

Seed imported to New Zealand from the United States of America is harvested in October and shipped to New Zealand in November - December. At this stage it is too late to be used by most commercial growers for the current seasons sowings. As a result the seed remains in storage until the following October when it is sold as 'new season's' seed. In reality such seed is at least 12 months old since harvest. Hence the relatively low incidence of A. zinniae associated with 'new season's' seed in New Zealand, compared with the high percentage of the

pathogen recorded as being present in seedlines overseas, may be due to a loss of viability of seedborne inoculum over the first 12 months since harvest. It is postulated that this reduction in disease incidence in zinnia seed lines by the ti they are sown in New Zealand is due to a rapid loss of viability of many conidia of the pathogen present on the seedcoat. That the fungus does not die out completely is shown by the fact that in 'new season's' seed for sowing in New Zealand some spores on the seedcoat and mycelium established in the tissues of the seed have been shown to retain their viability for as long as 2 and 3 years from harvest respectively. This is borne out by Jacks (1963) who has demonstrated that A. zinniae shows a far greater decline in viability up to one year from harvest than does the germination percentage of the seed, and the pathogen dies out completely in seed stored for 5 years from harvest.

C. CONTROL OF SEEDBORNE INOCULUM.

In the present study it has been conclusively proven that Alternaria zinniae may be associated with zinnia seed imported to New Zealand not only as conidia borne externally on the seedcoat but also as mycelium established within the testa and embryo. Yet there is the claim by Beaumont, Cleary & Bant (1958) that complete control of the disease in seedlines known to carry the pathogen can be effected simply by dusting the seed with Thiram (T.M.T.D.). This can only be explained by assuming that the seedlines used in their experiments were contaminated, but not infected with A. zinniae. Neergaard (1945), Kispatic (1951) and De Tempe (1959) all record incomplete control following surface sterilisation and it is concluded that in these cases both seed contamination and infection were involved.

In view of the fact that imported zinnia seedlines are consistently infected (as well as contaminated) it was decided to investigate the effectiveness under New Zealand conditions of those methods of seed treatment which could possibly be lethal to vegetative mycelium established within the seed.

I. PHYSICAL METHODS.

Physical methods of seed treatment have been widely used, primarily to kill pathogens deep in the seed (Hanson et al. 1961). These methods include hot-water and water-soak treatments, and ultraviolet, infra-red, X-ray and other kinds of irradiation. Dry heat has also been used. Only the hot-water and water-soak treatments have been shown to be practical. Accordingly these two methods were studied as possible treatments for the control of A. zinniae in zinnia seed.

HOT-WATER TREATMENT.

The most promising approach to zinnia seed disinfection seemed to be that described by Baker (1956), where effective control resulted from hot-water treatment at 125°F for 30 minutes, without causing any great reduction in the germination capacity of the treated seed. However, Beaumont, Cleary & Bant (1958) report that in their experience this treatment gave only moderate control and had a

severely depressing effect on germination. Prota (1960) studied hot-water treatment of zinnia seed at 51 to 52°C for 30 minutes and showed that in his experiments thermal treatment reduced percentage infection by only 28% and suppressed germination by 32.4%. As virtually all zinnia seed available in New Zealand is imported from the Northern Hemisphere and at the time of sowing is approximately 12 months old, it was thought that even if Baker's method did prove effective in New Zealand it would possibly reduce the germination capacity of the seed to a level which would render the method unacceptable to commercial nurserymen. Accordingly, investigations were undertaken to determine

1. Whether Baker's recommendation of hot-water treatment of seed at 125°F for 30 minutes eliminated the pathogen, and

2. The extent to which this treatment reduced the germination capacity of the seed.

This work was carried out as two trials, one conducted in the laboratory and the other within a commercial nursery.

MATERIALS and METHODS.

A. Laboratory Trial.

For this work 32 lines of seed which had given rise to infected seedlings when sown were used. The weights of seed used for each line varied, with the varietal size of the seed, as follows:-

4 gm. for large seeded varieties.	Giant Dahlia flowered, Giant Cactus flowered, Giants of California.
3 gm. for medium seeded varieties.	Cupid, Persian Carpet, Lilliput, Thumbellina
1 gm. for small seeded varieties	Linearis. Snowflake.

The seed from each line was placed on a square of muslin 5" x 5", the corners of which were gathered together and secured with a rubber band to form a bag. Different coloured bands allowed easy identification. The bags were placed in a wire mesh cage 7" x 5" x 4" and the cage immersed in a bath containing water held at 125°F (51.6°C) for 30 minutes. Entrapped air was expelled from

the bags by prodding with a glass rod. A thermostatically controlled temperature unit was used to keep the water at 125 $\pm$0.3°F and also to allow continuous circulation of the water in the bath. After 30 minutes immersion the cage was removed from the hot water and plunged into water which had been cooled with ice to 8°C. Each bag was kneaded with the fingers to ensure complete penetration of the cold water through the seed mass and so prevent any further action by the hot water on the seed. The bags were removed to cold running water (tap) for 10 minutes and the seed then spread out between two sheets of sterile blotting paper for 24 hours at room temperature to dry.

To determine the effect of the hot water treatment on germination, tests were carried out on each seedline both before and after treatment by the folded towel method at 25°C, interim and final counts being made at 3 to 7 days respectively.

To determine the effectiveness of hot water treatment in controlling the pathogen, a sample of each treated seedline was germinated on moist blotting paper in plastic trays in a Copenhagen tank and the percentage of seedlings which developed cotyledonary lesioning recorded. Results were taken at 10 days.

B. Commercial Nursery Trial.

This trial was conducted in a commercial nursery in Palmerston North during October and November 1963, using two lines of zinnia seed (Giant Dahlia Flowered and Lilliput) each of which, in preliminary tests, was found to give approximately 12% infected seedlings within 10 days from sowing. A 1 ounce sample of each line was hot-water treated at 125°F for 30 minutes, following the procedure previously described. Boxes of treated and untreated seed of each line were sown by the nurseryman, set out under glass for 8 days and then transferred to open frames until ready for transplanting. Care was taken to keep all boxes well separated to prevent the possibility of secondary spread of the pathogen between treated and untreated seedlings. At 10 days from sowing the percentage emergence in each box was recorded. Transplanting of seedlings from each of the four series

was carried out 22 days from sowing, the plants being pricked out into 18" x 12" boxes at the rate of 80 seedlings per box. The number of plants which developed lesioning were recorded at frequent intervals from 9 days after sowing. One lesion per plant was considered sufficient to record that plant as being diseased.

The germination capacity of each seedline both before and after treatment was determined using the folded towel method.

RESULTS and DISCUSSION.

A. Laboratory Trial.

Data on the effect of Baker's hot-water treatment method on seed germination and disease incidence are presented in Table 40. Table 41 records the range in germination percentage produced from seedline samples before and after hot-water treatment.

The results of germination tests before and after hot-water treatment are shown graphically in Figure 13.

a. Effect of hot-water treatment on seed germination.

Although no absolute conclusions could be made on the germination reduction likely to occur following hot-water treatment, the following trends were revealed:

1. Initially high germinating lines (over 85%) gave a germination percentage in the range 45 to 77% following hot-water treatment.
2. There was no relationship between interim and final germination percentages of untreated seed and the germination percentages obtained following hot-water treatment. That is, seedlines with a low interim and high final germination initially did not give lower germination percentages following hot-water treatment than high vitality lines which showed high interim and final germination counts.

Estimated vigour of line based on interim count	Initial germ. % 3 days	7 days.	Germ. % after hot-water treatment. 3 days	7 days.
High	87	91	27	45
Low	80	96	54	77
Moderate	82	91	50	57
High	66	71	22	44
Low	55	71	24	59
Low	51	70	33	69

FIGURE 13 EFFECT OF HOT-WATER TREATMENT ON SEED GERMINATION CAPACITY

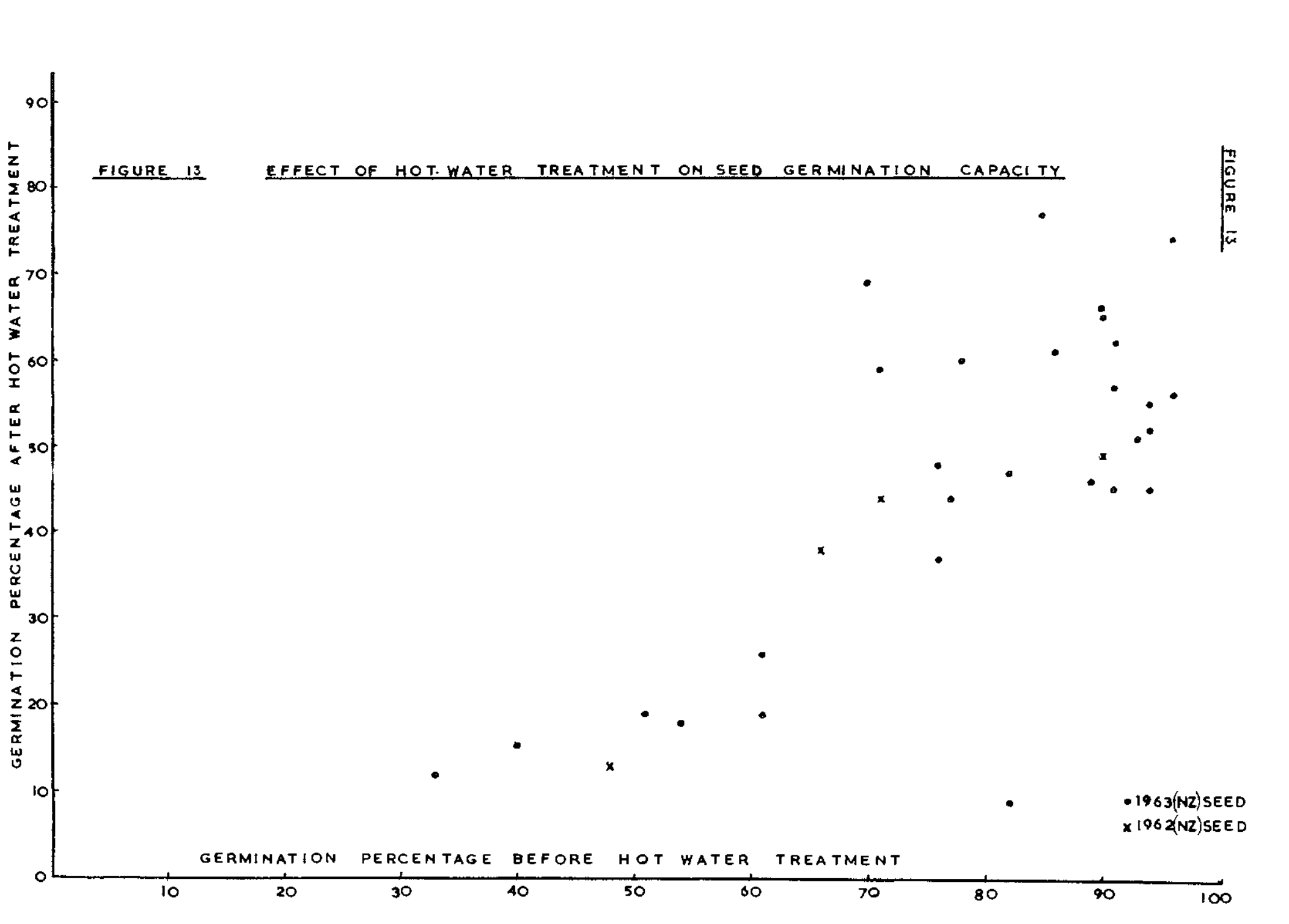


FIGURE 13

TABLE 40. EFFECT of HOT-WATER TREATMENT on SEED GERMINATION and DISEASE INCIDENCE.

Variety.	Initial Germn. % 3 days.	7 days.	% Cotyledonary lesioning at 10 days on Blotters.	Germn. after H/T. 3 days.	7 days.	% Cotyledonary lesioning at 10 days on Blotters.
GCF Cherrytime	80	96	11	54	74	0.5
California Giants	86	96	7	51	56	1.0
Super Giants	86	94	9	31	45	1.5
Lilliput Scarlet gem	86	94	4	39	55	0
GDF Polar Bear	83	94	5.5	44	53	0
Cupid	81	93	7	25	51	2.0
GDF Scarlet flame	82	91	16	50	57	2.5
GCF Dark Jewels	87	91	7	27	45	0
GDF Oriole	87	91	13	48	62	4.5
Lilliput Black Ruby	78	90	13	52	70	2.0
Thumbellina	76	90	7	50	71	2.5
+ Lilliput	80	90	15	34	49	3.5
Californian Giant	81	89	12	31	46	2.5
GCF Charrytime	83	86	8.5	49	61	1.5
Persian Carpet	63	85	5	48	77	0
Lilliput	52	82	5.5	36	47	1.5
Lilliput Purple Gem	70	82	5.5	3	9	0.5
GDF Polar Bear	68	78	7.5	45	60	1.0
Red Riding Hood	69	77	3	31	44	0
GCF Blaze	70	76	4	18	37	4.0
Thumbellina	70	76	12	24	48	2.5
Fantasy	55	71	11	24	59	6.0
+ Super Giants	66	71	18	22	44	2.5
Linearis Orange	51	70	6	33	49	1.5
+ GCF Dark Jewels	59	66	16	25	38	5.2

TABLE 40 (Ctd.)

Variety	Initial Germn. % 3 days.	7 days.	% Cotyledonary Lesioning at 10 days on Blotters.	Germn. after H/T. % 3 days.	7 days.	% Cotyledonary Lesioning at 10 days on Blotters.
Linearis Dwf.orange	58	62	6	12	19	1
GDF Gold Medal Hybrids	51	62	12	17	26	4.0
Creeping Zinnia	20	54	1	10	18	0
GCF Floradale Scarlet	37	51	6	12	19	1.5
+ Elegans pumila	43	48	18	5	13	3.5
Lilliput Valencia	18	40	7	8	15	1.0
Perennial snowflake	26	33	2	1	12	0

KEY: GDF = Giant Dahlia Flowered
GCF = Giant Cactus Flowered.

+ denotes 1962 seed. All other lines 1963 seed.

TABLE 41. RANGE IN GERMINATION PERCENTAGE PRODUCED FROM SEEDLINE SAMPLES BEFORE AND AFTER HOT-WATER TREATMENT.

Germination % before H.W.T.	Germination % after H.W.T.
90 - 96	45 - 74
82 - 86	(9)46 - 77 +
70 - 78	37 - 49
62 - 66	19 - 38
51 - 54	18 - 19
40 - 48	13 - 15
33	12

+ True range is 9-77% but excluding the germination reduction from one sample (82% - 9%) the range is 46% - 77%.

RECORD OF THE NUMBER OF SAMPLES IN EACH GERMINATION GROUP.

Range in Germ'n %	Total number of Samples in each Range of Germ'n % (Untrtd. seed)	Av. Germ'n in each grp.	Total number of samples in each range of Germ'n. % (H.W.T.seed).	Av. Germ'n in each group.
90-100	12	92.5	-	-
80-89	5	84.8	-	-
70 - 79	7	74.1	4	73.0
60 - 69	3	63.3	3	61.0
50 - 59	2	52.5	6	55.2
40 - 49	2	44.0	9	48.6
30 - 39	1	33.0	2	37.5
20 - 29	-	-	1	26.0
10 - 19	-	-	6	16.0
0 - 9	-	-	1	9.0

3. It was not possible to predict accurately the germination reduction due to hot-water treatment at 125°F for 30 minutes, except that the maximum drop was 50% in 31 of the 32 lines treated. In one line only a drop of 73% was recorded

The reason for the erratic results of hot-water treatment on germination reduction may be variations in seed moisture content prior to treatmen This effect was reported by Gloyer (1931) to explain inconsistent germination results following hot-water treatment of seed of Callistephis chinensis.

4. Seedlines with an initial germination of 90 - 95% showed a far smaller range of hot-water treatment effect on germination than did lines of lower initial germination of 30 - 60%.

b. Effect of Hot-water Treatment on Disease Incidence.

1. There was no apparent relationship between percentage germination and the disease incidence in seedlings either before or after hot-water treatment. Seedlines giving rise to seedlings with a high disease incidence were not necessarily low germinating lines and vica versa. This indicated that the presence of the pathogen had no depressing effect on seed germination percentage.

2. Initially diseased seedlines may still give rise to lesioned seedlings following hot-water treatment. These lesions were macroscopically identical with those produced on seedlings arising from untreated seed.

3. Hot-water treatment did not allow complete control of A. zinniae in seed lines in which the pathogen was borne internally. This was shown by the presence of lesions on the cotyledons of seedlings arising from hot-water treated seed. It is suggested that the 7 lines which were rendered disease free by hot-water treatment were lines in which the pathogen was externally associated with the seed, or present in the outer tissues of the testa only. In the remaining 25 lines lesions were found to be present in the embryo of the seed prior to hot-water treatment. This lesioning can be easily demonstrated by seed dissection.

B. Commercial Nursery Trial.

(a) Effect of hot-water treatment on seed germination.

The germination results obtained from treated and untreated samples of each line are compared in Table 42.

TABLE 42. EFFECT of HOT-WATER TREATMENT on SEED GERMINATION.

Seedline.	Germination percentage of seed.					
	Nursery sowing (10 days)			Folded Towel Method (7 days)		
	Untreated.	Treated.	Reduction %.	Untreated.	Treated.	Reduction %.
Giant Dahlia flowered	89	52	41.6	94	64	31.9
Lilliput	91	52	42.9	97	62	36.1

Average reduction due to hot-water treatment Giant Dahlia Flowered 42.3%
Lilliput 34.0%

These results show that treated samples sown under commercial conditions gave an average reduction in emergence of 42.3% compared with untreated samples. This was a somewhat greater reduction than had been recorded in samples tested for germination by the folded towel method in the laboratory. By the folded towel method the average reduction in germination following hot-water treatment was only 3% in each line. This discrepancy may have arisen from the dry soil conditions under which the samples were sown in the nursery.

(b) Effect of hot-water treatment on disease incidence.

Results of disease incidence as recorded by counts of the number of seedlings showing foliage lesioning are presented in Table 43, and graphically in Figure 14. Transplanting losses are also recorded in Table 43.

These results show the pronounced build-up of disease in seedlings from untreated seed due to initial inoculum acting as foci for the sporulation and secondary spread of the pathogen. In hot-water treated samples disease incidence showed only a slow increase and treated seedlines showed markedly less diseased plants 40 days drom sowing compared to untreated seedlines. Thus hot-water treatment allowed the production of a greater number of saleable plants when compared with the same number of seedlings arising from untreated seed.

CONCLUSIONS.

There appears to be no absolute relationship between initial germination percentages of seedlines and the germination capacity of seed following hot-water treatment. It is only possible to give tentative figures on the probable germination reduction likely to occur from hot-water treatment of zinnia seed.

In order for growers to treat seed by the hot-water method (125°F, 30 mins.) and obtain seed with a final germination of 40% or more, the germination percentage of the untreated line should be 70 - 80%. To obtain a germination following treatment of over 50% the initial germination should be over 85%.

While the hot-water treatment as advocated by Baker (1956) still allows many lines to produce lesioned seedlings on germination following treatment, the

FIGURE 14. DISEASE INCIDENCE IN SEEDLINGS ARISING FROM H.W.T. AND NON-H.W.T. SEED. - COMMERCIAL NURSERY TRIAL.

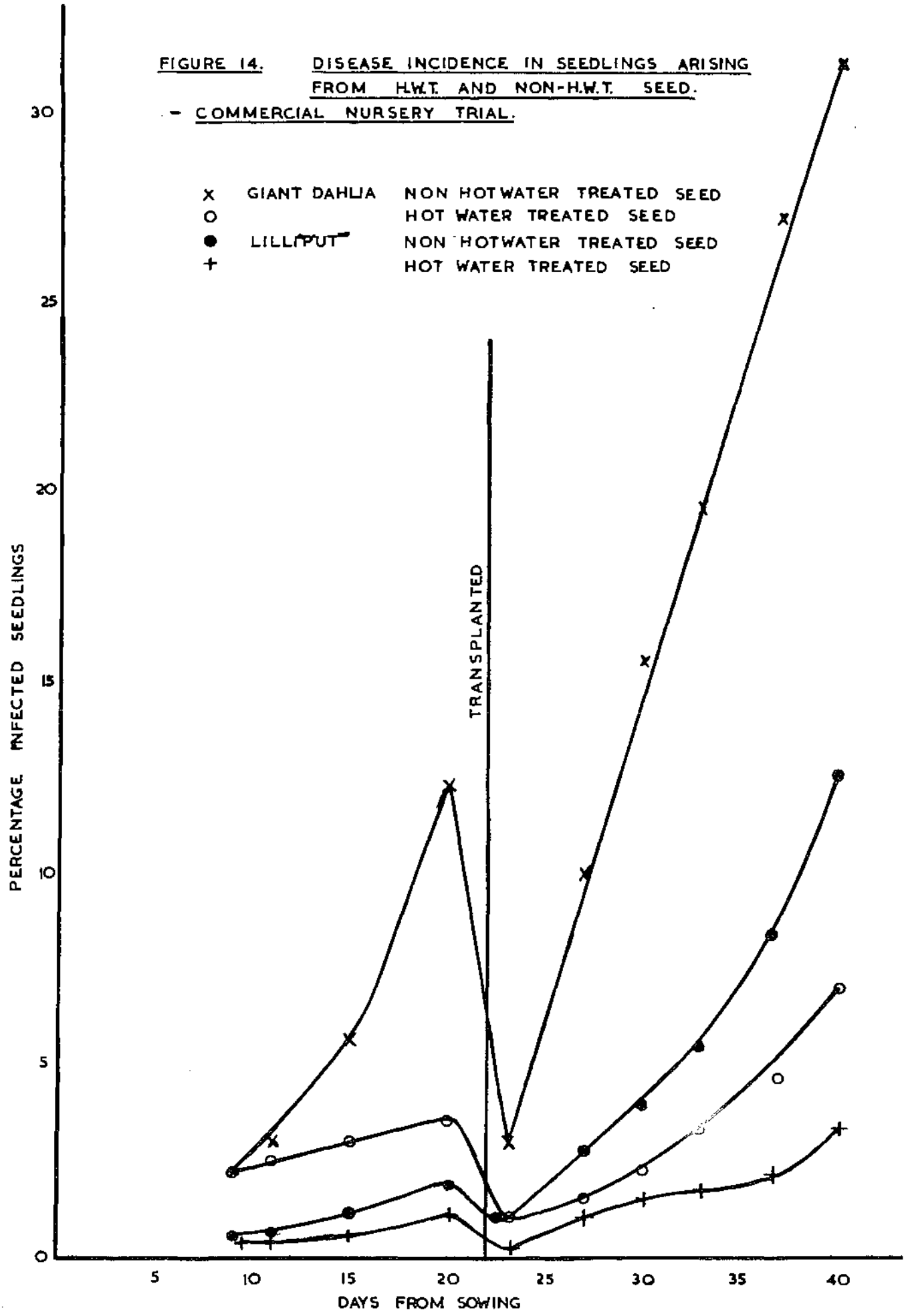


TABLE 43. DISEASE INCIDENCE in COMMERCIAL NURSERY TRIAL on EFFICIENCY OF HOT-WATER TREATMENT (H.W.T.)

Seedline.	Days from Sowing.	Number of Diseased Seedlings. Non H.W.T.	H.W.T.	Percentage Infection. Non H.W.T.	H.W.T.
Giant Dahlia Flowered.	9	28	14	2.2	2.2
	11	38	16	3.0	2.5
	15	72	19	5.8	3.0
	20	154	23	12.3	3.5
	22	TRANSPLANTED	80 Seedlings per box.		
	23	22	6	3.0	1.0
	27	69	9	9.6	1.5
	30	112	13	15.6	2.2
	33	146	19	19.5	3.2
	37	191	28	26.5	4.7
	40	224	40	31.1	7.0
Lilliput	9	9	4	0.5	0.5
	11	10	4	0.6	0.5
	15	20	4	1.1	0.5
	20	38	9	2.1	1.1
	22	TRANSPLANTED 80 Seedlings per box.			
	23	15	2	1.0	0.3
	27	42	8	2.9	1.1
	30	57	11	4.0	1.5
	33	79	13	5.5	1.8
	37	121	16	8.4	2.2
	40	182	24	12.6	3.3

TABLE 43 (Ctd.)

Seedline.	ORIGINAL SOWING. No.boxes.	No.seedlings 10 days.	TRANSPLANTED. No.boxes	No.seedlings.	Transplanting loss	% Loss.
Giant Dahlia Non.HWT	4	1,250	9	720	530 +	42.4
" " HWT	3	650	7.5	594	56	8.6
Lilliput Non HWT	5	1,850	18	1,440	410	22.2
" HWT	3	800	9	720	80	10.0

+ = 1 box of 300 seedlings from original sowing discarded due to disease.

Conclusions (ctd.)

method may be of use in allowing growers to produce more saleable plants from heavily diseased lines. The effect of hot-water treatment in suppressing the initial buildup of the disease appears most important in the early stages of plant growth (the first 15 days from sowing). This method may overcome the need for an intensive spray program during the first two weeks of growth when the seedlings are becoming established. Thereafter spraying may be employed to keep the disease to low levels until the plants are sold at 6 - 7 weeks.

Since zinnia seed is relatively inexpensive, the need for growers to sow a greater number of seeds to offset a germination reduction following hot-water treatment is more than compensated for by the ability of hot-water treatment to keep A. zinniae at a lower level than is possible with untreated seed. Hot-water treatment thus allows the production of a greater number of healthy plants for sal

In the light of previous experiments which had proved the presence of lesions on the embryo of ungerminated seeds it was thought that while these lesions would not be eradicated by thermal treatment this treatment may be capable of killing the fungus in such lesions.

Thus in a study of methods aimed at controlling A. zinniae by internal seed

disinfection it is postulated that the appearance of lesioned cotyledons on seedlings arising from treated seed does not necessarily prove the inadequacy of the treatment.

Many workers have based the success or failure of a particular seed control method for A. zinniae on the presence or absence of lesioned seedlings arising from treated seed (Neergaard (1945), Baker (1956), De Tempe (1959), Beaumont et al (1958) and Prota (1960). This assumption that the presence of cotyledonary lesions proves the inadequacy of the method as an absolute control may be incorrec Attempts to reisolate the pathogen from such lesions by high humidity treatment or by plating of lesioned tissue must be made to determine whether the fungus is capable of sporulation and vegetative growth from the lesioned area.

Accordingly studies were undertaken to test the validity of making numerical counts of lesioned seedlings grown from hot-water treated seed as a method of accurately determining disease incidence.

Unfortunately only 10 of the 32 lines used to test the effect of hot-water treatment on seed germination and disease incidence in the previous experiment were available in sufficient quantity to be incorporated in this study.

MATERIALS and METHODS.

Seeds were counted out for each of 10 lines of zinnia seed. Three hundred seeds per line were hot-water treated at 125°F for 30 minutes. The seeds were then placed on moist blotters in bakelite trays and germinated in a Copenhagen Tank at 25°C. Two hundred untreated seeds were also germinated in the same manner, as controls. After 7 days incubation those cotyledons in each line showing lesioning were removed. The lesioned cotyledons from untreated and treated samples of each seedline were cut medianally, ensuring that the lesion was bisected in the process. This allowed a cut surface across the lesion which could aid the fungus, if viable, to sporulate and also to act as a ready point of exit of the fungus for growth into culture media when cotyledons were plated to agar.

The bisected cotyledons were placed in muslin bags and the tops secured with rubber bands. The bags were washed vigorously in tap water for 4 hours and then opened and spread out on blotting paper for 1 hour to allow the tissue pieces to dry. Half the total number of tissue pieces from each sample were plated aseptically to prune agar and incubated at 28°C for 5 days. The remainder were placed on slides in petri plates lined with moist filter paper and incubated at 28°C for 4 days. Any fungal growth in culture arising from plated tissue was cut with a corkborer and placed on the inside of the lid of a petri plate. Sporulation if present was observed under a 40X binocular microscope after 48 hours incubation. The tissue pieces subjected to high humidity were examined after 4 days for sporulation under the binocular microscope.

Spores produced from both plated tissue and from tissue subjected to high humidity were placed on PDA slopes to determine viability.

The experiment was repeated on a further line of zinnia seed (Giant Dahlia Flowered Mixed) using exactly the same method but a larger quantity of seed (15gm or approximately 1,600 seeds).

RESULTS and DISCUSSION.

Results of the incidence of *A. zinniae* produced from lesioned cotyledons of hot-water treated and untreated seed are presented in Table 44, (Page 208).

These results show the marked reduction in disease incidence effected by hot-water treatment at 125°F for 30 minutes.

This reduction is shown in Table 44 (A) by the lower successful reisolation of the pathogen from lesions arising from hot-water treated seed and plated to agar, compared to lesions from untreated seed. In this experiment the reduction averaged 84.7% (31.3% to 4.8%). In 6 of the 10 lines treated, attempts to reisolate the fungus from lesioned cotyledons by plating was unsuccessful. It is assumed therefore that although hot-water treated seed of such lines gave rise to lesioned seedlings the fungus in the lesioned area was incapable of growth or sporulation.

The reduction in ability of the fungus to sporulate when lesions from hot-water treated seed were subjected to high humidity was 87.0% (13.1% to 1.7%). Of the 10 lines studied in Table 44 (A), the 5 lines which showed no growth on culture also showed an inability to produce spores when lesioned tissue was placed under high humidity conditions. In addition, of the remaining 4 lines which produced fungal growth from lesioned tissue on agar, 2 were incapable of spore production under high humidity.

Results are presented in Table 44 (B) for experimentation involving only one line of seed, but greater seed numbers, than used for each of the 10 lines previously studied. The effect of hot-water treatment at 125°F for 30 minutes on both germination and disease incidence in this line are summarised below:-

1. Germination reduction due to hot-water treatment (89% to 48%) was 46.1%.
2. Reduction in the viability of the fungus from lesioned cotyledons plated to agar was 51% (11.6% to 5.7%).
3. Reduction in sporulation capacity by the fungus from lesioned cotyledons subjected to high humidity was 77.5% (8.0% to 1.8%).

TABLE 44 A. EFFECT OF HOT-WATER TREATMENT OF SEED ON VIABILITY OF THE PATHOGEN IN COTYLEDONARY LESIONS ON SEEDLINGS.

Seedline	UNTREATED SEED.				HOT-WATER TREATED SEED.			
	% Lesioning 7 days 25°C.	No. Cotyl. Plated.	No. Showing A. zinniae.	% Recovery A. zinniae.	% Lesioning 7 days.	No.Cotyl. Plated.	No. Showing A. zinniae.	% Recovery A. zinnia
Lilliput Pompom	4.0	10	4	40.0	4.7	12	-	-
Giant Dahlia Fl. Mixed	7.6	22	6	27.5	8.4	16	-	-
G.D.F. Exquisite	6.2	19	6	31.6	7.4	18	-	-
G.D.F. Polar Bear	10.7	16	5	31.3	8.8	9	1	11.1
California Giants	8.0	8	3	37.5	5.1	8	-	-
Cherrytime	28.6	31	3	9.7	16.4	39	3	7.7
Cupid Mixed	3.2	8	3	37.5	3.0	3	-	-
+ G.C.F. Dark Jewels	13.2	12	2	16.7	10.1	7	1	14.3
Linearis Dwarf	6.2	6	3	50.0	3.1	3	-	-
Thumbelina Mixed	4.0	7	1	14.3	2.8	7	1	14.3
			AVERAGE	31.3			AVERAGE	4.8

	No. Cotyl. under High R.H.	No. Showing A. zinniae.	% Recovery A. zinniae.	No. Cotyl. under high R.H.	No. showing A. zinniae.	% Recovery A. zinniae.
Lilliput PomPom	12	2	16.6	10	-	-
Giant Dahlia Fl. Mixed	14	3	-	12	-	-
G.D.F. Exquisite	10	1	10.0	10	-	-
G.D.F. Polar Bear	12	1	8.3	9	-	-
California Giants	10	2	20.0	9	-	-
Cherrytime	21	1	4.8	35	1	2.9
Cupid Mixed	4	1	25.0	3	-	-
+ G.C.F. Dark Jewels	11	1	9.0	7	1	14.3
Linearis Dwarf	4	1	25.0	3	-	-
Thumbelina Mixed	8	1	12.5	6	-	-
		AVERAGE	13.1		Average	1.7

+ = 1962 Seed. All other lines 1963 seed.

G.D.F. = Giant Dahlia Flowered.

G.C.F. = Giant Cactus Flowered.

TABLE 44 (B,.

EFFECT of HOT-WATER TREATMENT of SEED on VIABILITY of the PATHOGEN in COTYLEDONARY LESIONS on SEEDLINGS.

Seedline Giant Dahlia Flowered. 10 gm. untreated: 15 gm. treated.

Germ. % 3d.	Germ. % 7d.	No.Cotyl. plated.	No.showing A. zinniae	% A.zinniae.	Germ.% 3d.	Germ.% 7d.	No.cotyl. plated.	No.showing A.zinniae	% A.zinniae
87	89	121	14	11.6	40	49	152	9	5.7
		No.cotyl. high R.H.					No.cotyl. high R.H.		
		75	6	8.0			110	2	1.8

CONCLUSIONS.

It appears that under New Zealand conditions, hot-water treatment as advocated by Baker (1956) is not a complete control for A. zinniae internally borne in zinnia seed. The method does, however,effectively reduce the number of cotyledonary lesions in which the fungus is viable and capable of sporulation. In this respect it probably has a favourable effect in increasing the length of time from sowing before secondary spread becomes important in seedboxes under commercial conditions.

This experiment supports the theory that the number of lesioned cotyledons arising from the germination of the treated seed is no direct indication of the success or failure of hot-water treatment in seed disinfection.

Because of the ability of hot-water treatment at 125°F for 30 minutes to control A. zinniae associated with zinnia seed in 6 of the 11 lines tested above, a short corollary experiment to try to relate disease control to the relative importance of surface and deepseated inoculum present with the seed was carried ou

The method employed was identical to that described in a previous experiment (page 181). Two lots of 200 seeds were sown for each line.

Results are presented in Table 45.

TABLE 45. RELATIVE IMPORTANCE of SURFACE and DEEP-SEATED INOCULUM ASSOCIATED with SEEDLINES USED in HOT-WATER TREATMENT EXPERIMENT.

Seedline.	% Infection in Untreated Seedline.	% Infection in Dusted Seedline	Reduction in Infection due to dusting.	Relative Importance of surface & deep-seated inoculum.	Partial (P) or Complete (C) control obtained after Hot-water treatmt.
Lilliput pompom mixed	3.5	0.0	3.5	3.5/0	C
Giant Dahlia Flowered mxd.	8.0	1.0	7.0	7/1	C
G.D.F. - Exquisite	3.5	0.5	3.0	3/0.5	C
G.D.F. - Polar Bear	5.5	5.0	0.5	0.5/5	P
California Giant Mixed	7.0	1.5	5.5	5.5/1.5	C
Cherrytime	8.0	4.0	4.0	4/4	P
Cupid mixed	3.5	0.5	3.0	3/0.5	C
G.C.F. Dark Jewels	4.5	3.5	1.0	1/3.5	P
Linearis Dwf.Orange	6.0	1.0	5.0	5/1	C
Thumbelina mixed	3.5	3.0	0.5	0.5/3	P
Giant Dahlia Flowered	15.6	5.2	10.4	10.4/5.2	P

The data from this experiment is too sparse for safe interpretation, but as it stands it indicates that hot-water treatment effects complete control in some seedlines and only partial control in others, dependent on the amount of deepseated inoculum. Seedlines in this experiment which contained low levels of internal inoculum were rendered disease free by thermal treatment at 125°F for 30 minutes. In seedlines with internal inoculum present at higher levels hot-water treatment was only partially effective in disease control.

Although no direct experimentation was carried out to determine the reason for the effectiveness of Baker's hot-water treatment recommendation in controlling the pathogen in seedlines with low infection and yet not controlling the pathogen in lines with higher levels of true seed infection, the following hypotheses are offered as a possible explanation.

It is postulated that the reason for the effectiveness of hot-water treatment at 125°F for 30 minutes in completely controlling A. zinniae in some seedlines and not in others is due to the precise location of the vegetative mycelium in the seed. Lines with mycelium of A. zinniae restricted to the testa tissues would be more amenable to complete control by thermal treatment than lines where the pathogen is present as mycelium deep in the embryo.

In this experiment it is postulated that in lines shown to be infected with relatively high levels of internal mycelium the inoculum is present within both the testa and the embryo. In lines with low levels of infection, however, the pathogen may be restricted to the testa tissues of the seed only, and at this site the pathogen would be more readily accessible to hot-water.

Variations in the depth of seed infection by A. zinniae may possibly occur as a result as one or both of the following:-

1. Suitability of environmental conditions for infection.

Environmental conditions in the standing crop during the period of development when true infection of the seed could occur is important in determining the ability of A. zinniae to infect the seed. Conditions of high moisture and humidity within the flowerhead of zinnia plants, combined with a suitable

temperature, would probably be required before seed infection could occur. The presence of water (whether caused by heavy dews or rain) lying within the compact flowerhead would act as a substrate for spore germination and may have an effect in softening the outer coat of the seed and allowing seed infection. Heavy rain prior to harvest could also carry inoculum from infected flowers into close contact with the seed surface.

In seed growing areas where environmental conditions are unfavourable for the pathogen to penetrate the seedcoat it is suggested that seed infection would be of minor importance, and that any infection which did occur under such conditions would be restricted to the testa tissues. In areas where conditions are favourable for seed infection the pathogen is postulated as being capable of penetrating the seedcoat and establishing as vegetative mycelium within both the testa and the embryo of the seed. Thus it is possible that in seed from areas where environmental conditions were unfavourable for seed infection the pathogen could be more amenable to control by thermal treatment than in seed from areas where environmental conditions had been favourable for seed infection.

2. Physical barrier of the seedcoat.

In crops where viable inoculum is not present in sufficient quantity to be capable of seed infection until late in the growing season when the seed is at the 'near mature' stage of development, it is conceivable that the hardened seedcoat may provide a physical barrier to penetration by the pathogen. Under such conditions mainly surface contamination would occur. If this physical barrier is of importance, it is postulated that any limited amounts of mycelial penetration of the seed which could occur, under suitable environmental conditions for spore germination and infection, would be confined to the outer tissues of the testa. Such inoculum in the testa would be readily accessible to thermal treatment.

Conversely, under conditions when environment was favourable for seed infection, when viable inoculum was present and where the seedcoat was still soft and hence providing no barrier to penetration by the pathogen, high levels of

infection of both testa and embryo may occur. Under these circumstances a seedline may show the presence of high internal inoculum in a seed dusting trial and yet hot-water treatment may effect only a partial control.

It should be emphasised that this hypothesis has been used to explain the results in this particular experiment only. It is not considered that it would be applicable in all circumstances.

The results to date show the ability of hot-water treatment at 125°F for 30 minutes to afford absolute control of the pathogen in some seedlines but not in others. It is concluded that the results of this present study support Baker's (1956) claim for complete control of A. zinniae by hot-water treatment at 125°F for 30 minutes, but only in seedlines where seed infection is of low incidence.

Some seedlines may contain deepseated inoculum up to 15%. Since these lines are not rendered disease free by Baker's hot-water treatment recommendation, further studies were carried out to try to find an absolute control for seedborne infection by A. zinniae in seedlines where internal inoculum is present at high levels.

Studies of the minimum time: temperature relationship required to kill the pathogen associated with zinnia seed by thermal treatment.

In order to determine the minimum time of hot-water treatment of seed required to effect absolute control of the pathogen in seedlines where a high level of inoculum is present within the seed, experiments were carried out with two lines of zinnia seed. These two lines had been shown by dusting and sowing experiments using the method outlined previously (page 181) to be 10 - 12% diseased. The first line (Giant Cactus Flowered Mixed) was shown by sowing trials to produce 12% diseased seedlings 10 days from sowing. Of this 12%, 5% was shown to be due to the presence of internal inoculum. In the second seed line (Giant Dahlia Flowered) 6.5% of the total disease incidence was attributable to internally seedborne inoculum.

1. Determination of the minimum temperature required to kill the fungus in seed by immersion in hot water for 30 mins. and the effect of this temperature on seed germination.

MATERIALS and METHODS:

The above two lines of zinnia seed were used in this experiment, each treatment required 5 gm. of each variety. Temperatures of immersion ranged from 125°F to 131°F. at 1° intervals. Immersion time was constant at 30 minutes. After treatment at each temperature seeds from each sample were plated to prune agar (10 seeds per plate) and incubated at 28°C for 5 days. The remainder of the seed from the Giant Cactus Flowered line only was placed on moist blotters in Copenhagen Tanks and incubated at 25°C for 7 days. The diseased cotyledons arising from germination of treated seed on blotters were removed and cut longitudinally through the lesioned area. Half of each cotyledon was washed for 6 hours in running tap water, plated to prune agar and incubated at 28°C for 5 days. The remainder was given high humidity treatment at 28°C. for 4 days. Identification of fungal growth was carried out under a binocular microscope by identification of the spores formed in culture or on host tissue.

The germination percentage of both lines was determined by the folded towel method before and after treatment.

RESULTS.

Data on germination percentage and disease incidence in seed samples following treatment are presented in Table 46.

CONCLUSION.

Complete control of *A. zinniae* in seed was achieved by immersion in hot water at 129°F for 30 minutes. The thermal death point of the fungus was found by plating treated seed, by plating cotyledonary lesions on seedlings from treated seed and by high humidity treatment of lesions on seedlings grown from treated seed. This temperature is 4°F (2.2°C) higher than the temperature treatment advocated by Baker & Davis (1950) and Baker (1956) as an absolute control.

Table 46. GERMINATION PERCENTAGE and DISEASE INCIDENCE in SEEDLINGS TREATED in HOT-WATER at TEMPERATURES from 125-131°F for 30 MINS.

GIANT CACTUS FLOWERED MIXED. Plated seed (100 seeds per treatment).

Treatment	Germin. % 3 days	7 days.	No.fungal growths 5d.	No. A.z. colonies.	% A.z.
Untreated	81	87	38	8	21.1
125°F 30m.	34	36	15	2	13.3
126°F 30m.	26	35	15	4	26.7
127°F 30m.	25	32	21	2	9.7
128°F 30m.	15	20	16	2	12.5
129°F 30m.	10	15	32	0	0
130°F 30m.	8	14	26	0	0
131°F 30m.	4	7	30	0	0

SEED on BLOTTERS (200 seeds per treatment.)

Temp.°F.	%lesions	No.plated ½ cotyls.	No.fungal growths 5d.	No.A.z. colonies.	%A.z.	No.High RH. ½ cotyls.	No.fungal growths 4d.	No. A.z. col.	% A.z.
Untreated	17.4	50	24	4	8.0	50	17	5	10
125	9.3	26	23	3	11.5	25	10	2	8.0
126	8.6	18	9	1	6.0	18	11	2	11.1
127	7.3	15	8	1	6.7	15	8	1	6.7
128	6.7	8	8	1	12.5	10	4	1	10.0
129	8.9	8	8	0	0	8	4	0	0
130	7.2	9	5	0	0	10	6	0	0
131	5.9	6	5	0	0	6	2	0	0

GIANT DAHLIA FLOWERED MIXED Plated seed.

Temp.°F.	Germ. % 3d.	7d.	No. seeds plated.	No. fungal growths.	No. A.z. Colonies.	% A. zinniae.
Untreated	88	92	250	108	34	13.6
125	46	51	252	78	11	4.4
126	34	43	250	69	8	3.2
127	32	38	256	81	4	1.6
128	24	32	253	77	5	1.9
129	21	28	260	59	0	0
130	16	24	250	62	0	0
131	8	15	253	50	0	0

2. Determination of the minimum time of hot-water treatment at 129°F required to kill the fungus in seed and the effect of treatment at minimum time and temperature on seed germination.

MATERIALS and METHODS.

The seedline used in this experiment was the same line of Giant Cactus Flowered Mixed used in Part A. Samples of 5 gm. of seed were placed in each of 13 muslin bags and the top of each bag was loosely tied. The bags were immersed in hot water at 129°F for periods ranging from 10 to 30 minutes. They were then removed to running tap water for 10 minutes and the seed placed on sterile blotters for 48 hours to dry. Approximately 250 seeds were plated to prune agar and the plates incubated at 28°C for 4 days. Untreated seed was also plated to prune agar for comparative purposes. The fungal growths arising from the seed were cut with a corkborer and incubated for 48 hours at room temperature before positive identification by spore characteristics was made. Seed from each treatment was germinated by the folded towel method at 25°C, counts being made at 3 and 7 days.

RESULTS. Results are presented in Table 47.

TABLE 47. MINIMUM TIME of TREATMENT at 129°F REQUIRED to CONTROL A. ZINNIAE in SEED, and EFFECT OF THIS TREATMENT on GERMINATION PERCENTAGE.

Time of Immersion at 129°F in mins.	Germin'n % 3 day	7 day	No. seeds plated.	No. seeds with Fungal Growth	No. seeds with A. zinniae.	% A. zinniae.
Untreated	84	89	260	69	39	15.0
125°F, 30 min.	61	67	250	45	14	5.6
10	33	43	257	42	9	3.5
12	31	43	261	25	6	2.3
14	22	29	266	18	6	2.3
16	25	27	218	11	4	1.8
18	17	23	256	27	3	1.2
20	16	21	256	15	2	0.8
22	14	18	285	22	1	0.4
24	13	16	274	23	0	0.0
26	10	14	246	15	0	0.0
28	6	8	294	26	0	0.0
30	5	7	263	18	0	0.0

CONCLUSION.

From these results it is concluded that immersion of seed in hot-water at

129°F for 24 minutes is sufficient for complete control of A. zinniae in lines containing a high proportion of deepseated inoculum. These conditions differ from those recommended by Baker (1956) by 4°F and 6 minutes. Unfortunately the germination of the seed under these conditions is so low (a reduction of 82% in the sample used in this experiment) that the use of this method for absolute control of the fungus is not considered practical.

Tests have shown the ineffectiveness of Baker's recommended hot-water treatment of 125°F for 30 minutes as an absolute control of A. zinniae in zinnia seed, and also the severe reduction in the germination of seed exposed to the minimum temperature: time combination required to kill the fungus in seed (129°F, 24 min.)

Accordingly, work was carried out to determine the effect of combining Baker's method with a soaking of the seed in water prior to treatment. It was thought that this pre-soaking might increase the susceptibility of the fungus to thermal treatment.

3. Effect of soaking seed prior to hot-water treatment as a possible method of controlling A. zinniae internally borne in zinnia seed.

Jensen 1888 (Mundkur 1949) working on the control of loose smut of wheat caused by Ustilago tritici, found that if dormant mycelium in the seed was induced to germinate it became vulnerable to the application of heat, by which means it could be killed. The dormant mycelium itself was very resistant to heat. On this basis it was decided to determine the effect of soaking zinnia seed prior to hot-water treatment at 125°F for 30 minutes as a possible control of internally borne A. zinniae.

MATERIALS and METHODS.

In this trial 5 gm. of each of two seedlines was used for each treatment. Seed was soaked in an open 250 ml. Erlenmeyer flask in 100 ml. distilled water at 28° C. for periods varying from 1 to 12 hours. The seed was then tipped from

the flasks into a wire mesh strainer (1mm^2 mesh) and allowed to drain for 10 minutes. After bagging in a 5" square of cheesecloth, each sample was placed in a thermostatically controlled water bath at 125°F. After 30 minutes the bags were removed and placed under running tap water for 10 minutes. The seed was then removed from each bag and placed on sterile blotting paper to dry for 6 hours. To reduce the risk of aerial contamination a further sheet of sterile blotting paper covered the seed during the drying process.

After drying, 200 seeds were removed from each sample for a germination test. Seeds were plated aseptically to prune agar (20 seeds per plate) and incubated at 28°C for 5 days. Untreated seed and seed subjected to hot-water treatment at 125°F for 30 minutes but not pre-soaked, were used as controls. Fungal growths originating from seeds were cut with a corkborer and the agar circles incubated on the bottom of a petri plate lid at room temperature for 48 hours. Identification of fungi present was carried out under a binocular microscope by reference to spore characteristics.

RESULTS.

Seed plating results following treatment are presented on Table 48:

TABLE 48. SEED PLATING RESULTS OBTAINED FOLLOWING SOAKING OF SEED AND HOT WATER TREATMENT AT125°F FOR 30 MINUTES.

SEEDLINE 1 = GIANT DAHLIA FLOWERED MIXED. Seed soaked in 100 ml. distilled water 100 seeds per treatment plated to prune agar.

Treatment.	Germination % 3 day.	7 day.	No. with fungal growth 5 days.	No.colonies A. zinniae.	% A. zinniae.
Untreated	71	72	75	13	13.0
H.W.T. only	32	37	28	3	3.0
P1. H.W.T.	19	24	24	6	6.0
P2. H.W.T.	16	20	39	5	5.0
P4. H.W.T.	21	27	38	3	3.0
P6. H.W.T.	21	26	41	2	2.0
P8. H.W.T.	16	22	38	2	2.0

P1 - indicates pre-soak of 1 hour duration.
% A.z. is based on the total number of seeds plated.

TABLE 48 (Ctd.)

SEEDLINE 2. = GIANT DAHLIA FLOWERED GOLD MEDAL HYBRIDS.

Seed soaked in 100 ml. water.

Treatment	Germin'n. % 3 day.	7 day.	No.with fungal growth. No.seeds plated.	5 days.	No.colonies A. zinniae.	% A. zinniae.
Untreated	80	91	261	102	44	16.9
H.w.T.only	68	70	256	52	11	4.3
P1 H.w.T.	62	71	250	50	11	4.5
P2. H.w.T.	59	68	292	55	7	2.4
P4. H.w.T.	60	65	244	31	6	2.5
P6. H.w.T.	58	65	237	28	8	3.1
P8. H.w.T.	43	52	224	27	4	1.8
P10. H.w.T.	36	40	211	45	3	1.4
P12. H.w.T.	31	37	219	26	4	1.8

DISCUSSION.

As with the hot-water treatment alone, the germination percentage reduction in both lines of seed was variable and dependent on initial germination. That is, the seedline with the higher initial germination was less adversely affected by pre-soaking and hot-water treatment than the lower germinating line. This variability was probably due to the effect of the hot-water treatment rather than the pre-soaking of the seed.

Disease incidence as shown by the percentage recovery of the pathogen from treated seed plated to prune agar showed some reduction with increased length of pre-soak period. Even after a soaking period of 12 hours and subsequent hot-water treatment, however, the pathogen was still capable of growth from the seed into culture media.

CONCLUSION.

The use of a seed soak prior to hot-water treatment reduced the disease incidence compared to hot-water treatment alone, but the method did not allow complete control. At pre-soaking periods of 12 hours the germination of the seed was so adversely affected that the use of the method would be considered impractical on a commercial scale.

Baker's (1956) recommendation for hot-water treatment of zinnia seed at 125°F for 30 minutes has been advocated as a complete control of seedborne inoculum of A. zinniae, and has proved successful on a commercial scale in the United States of America. The present study, however, indicated that the method did not give complete success when used on zinnia seed in New Zealand. Seed used in New Zealand is at least 12 months old when sown, and because of this age effect the germination capacity of the seed is greatly reduced by hot-water treatment. This would tend to make the use of the hot-water treatment unacceptable to many commercial nurserymen.

Results in the present study indicated that treatment of seed in water at 125°F for 30 minutes gave absolute control of A. zinniae in some seedlines but not in others, the variability being postulated as being due to the precise location of the vegetative mycelium in the seed. Seedlines in which mycelium of the pathogen was restricted to the seedcoat tissues would be more amenable to complete control by thermal treatment than lines where the pathogen was present as mycelium deep within the seed embryo. Seedlines containing high levels of seed infection were not rendered disease free by Baker's hot-water treatment recommendation. In such lines the minimum temperature: time relationship for hot-water treatment of 129°F: 24 minutes was found to be necessary to control deep-seated inoculum of A. zinniae. At such a temperature: time ratio, however, germination percentage was so adversely affected as to make the use of this treatment of no practical value.

The use of hot-water treatment of zinnia seed at 125°F for 30 minutes, although not successful as an absolute control of A. zinniae, was shown to be very effective in reducing the initial build-up of the disease in commercial plantings and hence may be of use in reducing the number of sprays required to control the disease in the early stages of seedling development.

PLATE 18. Partially germinated seedlings - seedcoat removed from cotyledons. Lesions already present on developing cotyledons.

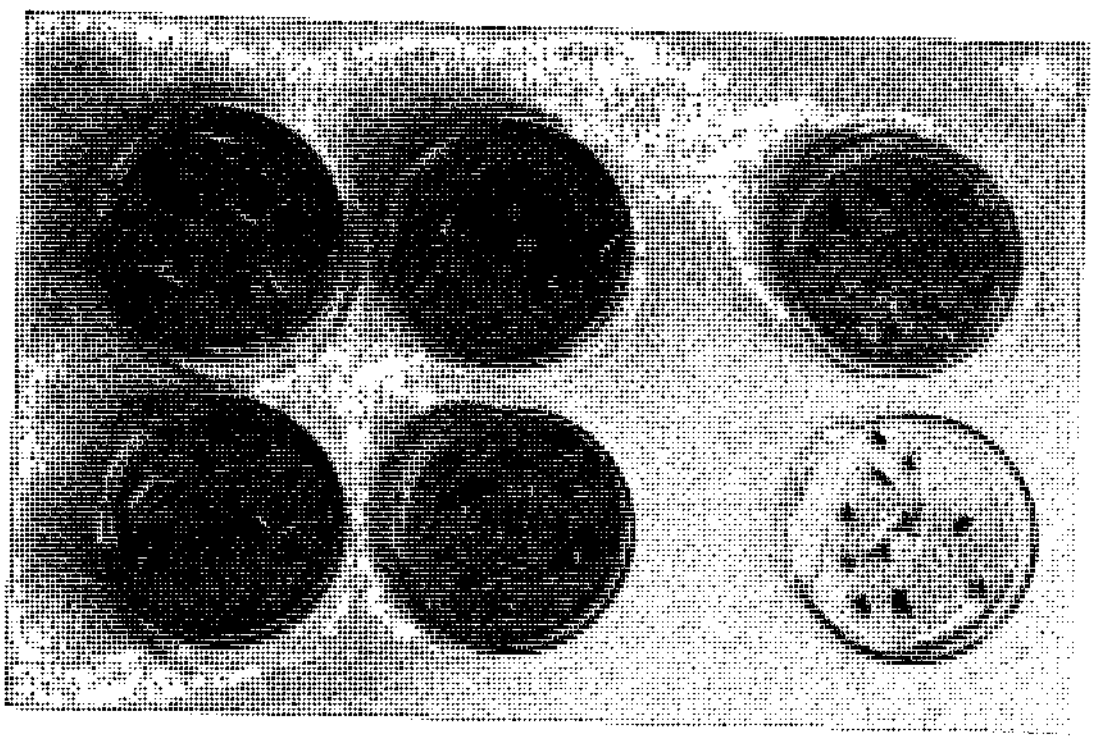


PLATE 19. Effect of temperature of hot-water treatment on viability of seedborne inoculum (30 min. immersion).
Top: Untreated 127°F. 129°F.
Bottom: 125°F. 128°F. 131°F.

WATER-SOAK METHOD.

Because the method of Baker (1956) of hot-water treatment of seed had not giv absolute control in all seedlines of Zinnia elegans under New Zealand conditions, an alternative method was sought which would inactivate the pathogen without undul reducing germination of the seed.

Tyner (1952) and Army (1957) have both described tests in which both loose smut of barley and wheat were sucessfully controlled by soaking a given weight of seed in a known quantity of water. This work is of special interest because the inciting fungi, Ustilago nuda Jens. and U. tritici Pers. respectively, are borne within the seed and therefore not easily inactivated. Because of the simplicity and success of water-soaking in control of these cereal diseases this method was investigated as a possible alternative to hot-water treatment for the control of A. zinniae internally borne in zinnia seed.

MATERIALS and METHODS.

Seed used in this study was a naturally infected line of Giant Dahlia Flowered with a final germination at 7 days of 80%. The relative importance of surface and deep-seated inoculum in this seedline was determined using the method previously described (page 181). Of the 15% disease incidence shown by cotyledon lesioning 10 days from sowing, 5.5% was due to seed infection.

A ratio by weight of one part seed to 10 parts distilled water was used in the soaking treatment. Usually 5 grams of seed (approx. 550 seeds) and 50 mls. of water were placed in open 250 ml. Erlenmeyer flasks and held at 28°C for periods of treatment ranging from 1 to 132 hours. Following each soaking treatment seeds were removed from the liquid and allowed to drain for 5 minutes on a 1 mm. mesh screen before being dried on sterile blotting paper at room temperature for 48 hrs. The liquid from the flask in each case was poured into a 100 ml. measuring cylinder to record the amount of water absorbed by the seed during the soaking process.

Determination of disease incidence in seed following the above treatments

was carried out in two ways, as follows:-

A. From each treatment approximately 250 seeds were transferred aseptically to plates of prune agar (20 seeds per plate) and incubated at 28°C for 4 days. The number of fungal and bacterial colonies arising from plated seed were recorded, and portions of the fungal colonies cut out with a 5 mm. corkborer. These latter were transferred to the underside of a petri dish lid and incubated at room temperature for 48 hours, at which time identification of the colonies was made on the basis of conidial characteristics.

B. Approximately 125 seeds from each treatment were sown in sterilised soil from the glasshouse. Lesioned cotyledons from seedlings were removed 14 days after sowing. These were washed for 6 hours in running tap water in the laboratory, dried between two sheets of sterile blotting paper for 2 hours and plated aseptically to prune agar. All plates were incubated at 28°C for 5 days. Fungal colonies arising from the lesioned tissue were cut out with a 5 mm. corkborer and the agar discs incubated in a petri dish at room temperature for 48 hours. Identification of the fungi present was made by reference to spore characteristics.

The effect of the various periods of soaking on germination capacity of the seed was determined by the folded towel method at 25°C.

Studies were also made of the effect the steep liquid and its components had in inhibiting spore germination. The seed soak liquid (25 ml.) was steam distilled and the distillate (15 ml.) and the non-volatile fraction (10 ml.) each made up to 25 ml. with sterile distilled water. The spores used in these studies were removed from the surface of a 10 day culture of *A. zinniae* which had been induced to sporulate under ultraviolet light.

A camelhair brush was used for transferring spores. Conidia were placed in the substrate on the surface of a glass slide and incubated at 28°C. Germination counts were made at 2, 4, 6, 8, 9 and 20 hours. Comparison of percentage germination in all cases was made against spores mounted in distilled water and incubated at the same temperature. Determination of germination percentage, germ tube length

and maximum number of germ tubes per spore were made for each substrate. Spores were considered to have germinated when the sides of the germ tube became parallel

RESULTS and DISCUSSION.

Results of disease incidence in soaked seed plated directly to agar, and the effect of soaking treatments on seed germination are recorded in Table 49 and graphically in Figure 15.

TABLE 49. DISEASE INCIDENCE in SEED TREATED by the WATER SOAK METHOD and PLATED to PURE AGAR.

Duration of water soak.	Water Absorbed by seed. (ml.)	Germination Percentage. 3 days.	7 days.	Number seeds plated.	Number seeds with fungal growth.	Number seeds with Bactl. growth.	Number A.zinniae.	% A.zinniae.
Untreated	-	78	80	258	96	50	40	15.5
8 hours.	15	76	79	257	99	74	28	10.9
12 "	20	70	74	247	70	71	18	7.3
16 "	22	69	74	250	55	74	21	8.4
24 "	22	71	73	250	64	79	22	8.8
36 "	21 +	68	73	252	56	72	13	5.2
48 "	21	61	67	257	69	77	11	4.3
60 "	20	51	60	255	33	67	3	1.2
64 "	22	46	55	165	34	67	3	1.8
132 "	27 ++	19	22	250	39	77	5	2.0

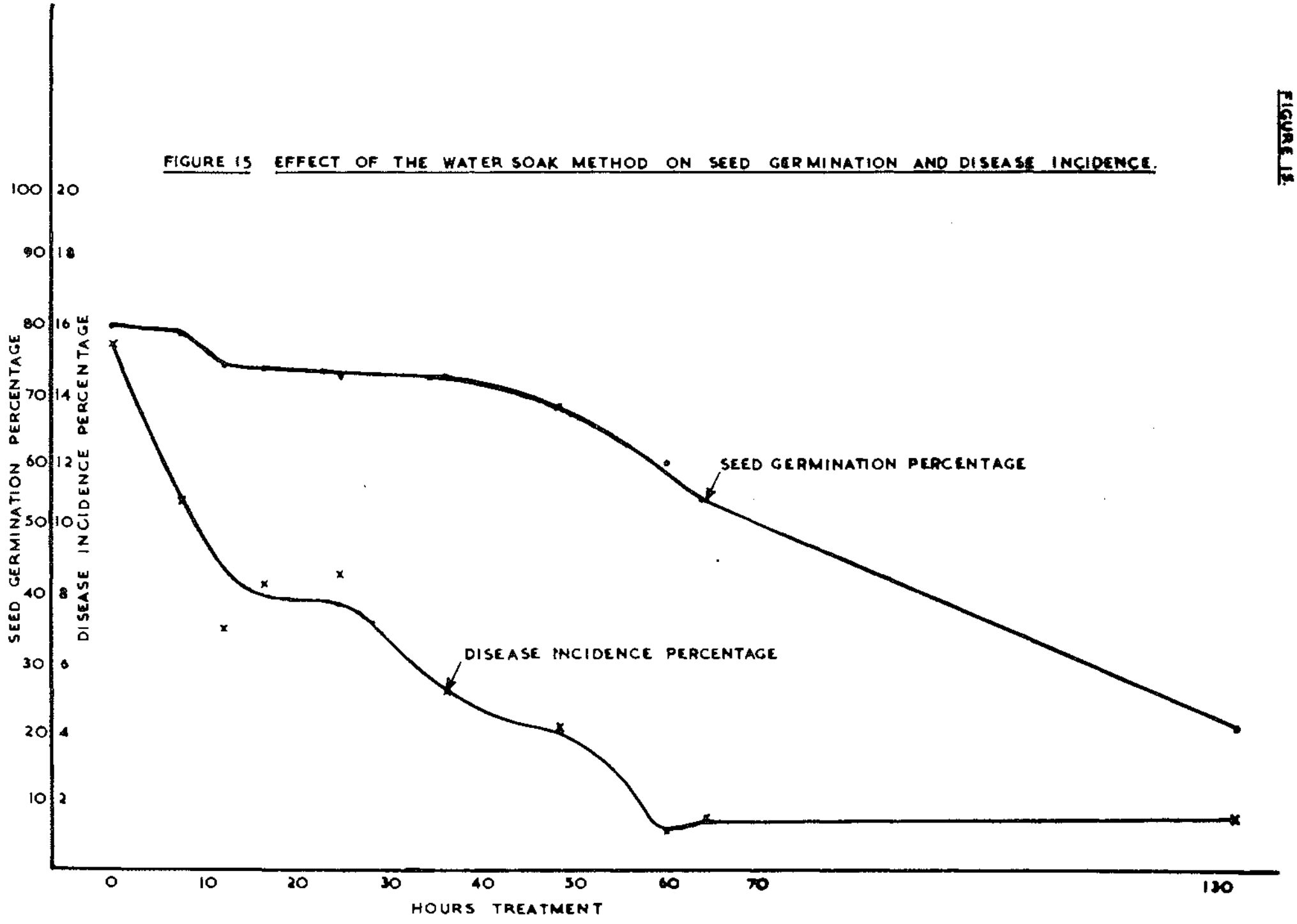
\+ = First evidence of foul odour at 36 hours.

++ = Surface scum present on steep liquid.

The water soak method as applied to the control of A. zinniae.in zinnia seed did not eliminate the pathogen after soaking periods up to 132 hours, but did greatly reduce disease incidence. This was shown by a reduction of A. zinniae from plated seed from 15.5% in the untreated line to 1.2% after a water soak of

FIGURE 15

FIGURE 15 EFFECT OF THE WATER SOAK METHOD ON SEED GERMINATION AND DISEASE INCIDENCE.



60 hours. The soaking method did not appear to depress the incidence of molds such as Penicillium spp and Mucor associated with the seed. The method, however, did have a reducing effect on the presence of saprophytic fungi such as Alternaria tenuis on the seed. A soaking period of 132 hours reduced the incidence of A. tenuis in plated seed to 2.4% compared with 13.2% in the untreated seedline.

Some workers (Army and Leben 1956, and Leben, Scott and Army, 1956, have proposed the theory that bacterial action is responsible, at least in part, for the effectiveness of the water soak method in the control of seedborne diseases. In the present study there was a marked increase in the number of bacterial colonies associated with plated seed following treatment. At a soaking period of 48 hrs the bacterial colonies growing from plated zinnia seed increased from 19.4 to 30.0 per 100 seeds. In this respect these results may support the above theory. It is therefore suggested that as a result of soaking seed in water there is an increase in the bacterial population, which population in some manner may inactivate A. zinniae.

In this study a water soak period of 60 to 64 hours appeared to be the most effective in causing reduction of disease. Further, at these periods of immersion the germination capacity of the seed was not severely reduced. This was shown by a percentage reduction of A. zinniae from 92.3% (15.5 to 1.2%) and a germination reduction of only 37.5% (80 to 50%) at a soaking period of 60 hours, compared to untreated seed.

The percentage of A. zinniae present when lesioned cotyledons arising from treated seed were plated to prune agar are presented in Table 50.

TABLE 50. DISEASE INCIDENCE from LESIONED COTYLEDONS ARISING FROM SEED TREATED by the WATER SOAK METHOD.

Hours Treatment	Number Seeds sown.	Number Emerged	Number Lesioned Cotyledons	Number Cotyledons Plated.	Number showing A. zinniae.	% A. zinniae.
Untreated	150	127	38	72	32	44.4
8	104	72	20	40	14	35.0
12	116	73	18	36	11	30.6
16	119	84	23	42	10	23.8
24	132	116	27	52	11	21.2
36	108	70	14	28	6	21.4
48	112	69	15	29	4	13.8
60	122	72	19	37	3	8.1
64	117	59	13	26	3	11.5
132	100	27	9	18	1	5.6

The fact that the water-soak method did not allow absolute control of A. zinniae in zinnia seed was shown by the ability of the fungus to grow out from lesions on seedling cotyledons arising from treated seed, when such lesions were plated to agar. As the results of direct plating of seed have already shown, a soaking period of 60 hours appears to give the most marked reduction in disease incidence. This is shown in Table 50 by only an 8.1% recovery of the fungus from lesioned cotyledons arising from seed soaked in water for 60 hours. Compared with the 44.4% reisolation success obtained from lesioned cotyledons arising from untreated seed, the 8.1% figure after 60 hours soaking represents a reduction in successful reisolation of the fungus of 81.8%. Thus the water-soak treatment has a marked effect in reducing fungal viability in lesioned cotyledons arising from treated seed.

The inhibitory effect of the steep liquid and its components on spore

germination are recorded in Table 51.

TABLE 51. EFFECTS of the WATER-SOAK LIQUID AND ITS FRACTIONS on SPORE GERMINATION CAPACITY.

A. - Average germination percentage obtained by incubating spores in the steep liquid, distillate, and non volatile fractions at 28°C.

Substrate.	Hours incubation.					
	2	4	6	8	9	20
Whole seed soak liquid	2.3	12.4	20.3	28.7	31.0	35.3
Distillate only	2.7	22.7	24.0	30.3	32.0	45.7
Non volatile fractions only	11.3	69.0	82.3	90.3	95.0	N.R.
Distilled water (CONTROL)	55.3	80.7	95.0	96.3	N.R.	N.R.

Germination % figures are the average of three random counts of 100 spores. N.R. = Not recorded.

B. - Spore percentage germination and germ tube development at 8 hours incubation in the steep liquid and fractions at 28°C.

Substrate	Germination %	Maximum Germ Tubes per Spore.	Maximum length of Germ Tubes +	Range in Germ Tube number per Spore.
Seed soak liquid (pH 7.5)	28.7	3	4	1 - 2
Distillate (pH 6.8)	30.3	5	2.5	1 - 3
Non Volatile fraction (pH9.8)	90.3	7	12	1 - 6
Distilled water (CONTROL) (pH 6.8)	96.3	13	14	8 - 10

+ = Maximum germ tube length is compared with maximum spore body width.

These results show that some component of the steep liquid which is retained in the volatile fraction following steam distillation has a marked inhibitory effect on spore germination. This inhibitor not only depresses the rate of spore germination by approximately 70% at 8 hours compared to spore germination in

distilled water, but also decreases the number of length of germ tubes produced per spore.

CONCLUSIONS.

It is concluded that soaking a ratio by weight of one part seed to ten parts of distilled water at 28°C for periods of 60 hours or more is effective in reducing disease incidence to low levels. In this respect it is at least as effective as the hot-water method previously described. The water-soak method also has the advantages of not being as severe as hot water treatment in reducing seed germination capacity, and of being extremely simple to use. It should be emphasised, however, that the water-soak method was not found to be effective as an absolute control of A. zinniae associated with seed of Zinnia elegans as internal mycelium.

II. CHEMICAL METHODS.

Because of the ineffectiveness of the physical methods of internal seed disinfection studied, some chemical methods were examined to determine their effect in controlling A. zinniae in zinnia seed.

Chemical methods are the most commonly used techniques of treating seeds to control internally and externally borne pathogens. A wide variety of chemicals are available for this purpose, many of which possess fungicidal and/or bactericidal properties, and may be applied as dusts or liquids.

SURFACE SEED DRESSINGS.

Since A. zinniae has been demonstrated to be internally as well as externally associated with zinnia seed (page 174) the application of chemical dusts to the seed was considered to be unlikely to control seedborne inoculum of the pathogen. Although surface seed dressings may be effective in killing conidia of the pathogen adhering to the seed surface it was thought that mycelium within the seed would not be controlled by such treatment. This viable internal mycelium would still allow seed infected with A. zinniae to act as initial foci for the buildup of the disease in seedlings arising from sowings of infected seedlines.

Overseas experimentation has shown some confusion regarding the efficiency of surface seed dressings in controlling seedborne inoculum of A. zinniae. Beaumont et al. (1958) report success in the control of seedborne A. zinniae by dusting seed with either 50% Thiram or Ceresan+. Such seed treatments were demonstrated by Beaumont et al. (loc. cit.) to effect complete control of A. zinniae. Negating this work, however, are the results obtained by Neergaard (1945), Mispatic (1951) and De Tempe (1959), all of whom found only partial control of the pathogen by seed dusting.

Accordingly, a trial in a commercial nursery was carried out using two seed dusts (Fernasan and Ceresan) to ascertain whether or not these materials were effective in reducing the number of diseased seedlings arising from plantings of diseased seed.

+ = Ceresan is the trade name of an organic mercury seed dressing which contained 1.5% mercury.

Materials and Methods.

The seedline used was a line of variety Giant Dahlia Flowered with an initial germination of 82%. Preliminary experiments showed that this line contained both surface and internal inoculum of A. zinniae, the relative importance of each source being 6/15 (see method page 181) Three 1 oz. lots of seed were used; one lot was dusted with Fernasan, one was dusted with Ceresan, and the third lot was untreated.

All three seedlots were sown by a commercial nurseryman under glass on the same day and the seedboxes were kept separate during the trial period to prevent any secondary spread of the pathogen between treatments.

The number of seedlings emerging were recorded 7 days after sowing and the germination of the untreated and dusted portions of the seedline was also assessed by the folded towel method in the laboratory. Counts of the number of diseased seedlings arising from each lot of seed were made at frequent intervals. Twenty-one days after sowing, 100 lesioned leaves from each treatment were removed to the laboratory. The leaves were washed in running tap water for 6 hours and then bisected through the lesioned area. One half of each lesion was plated to prune agar and the other half held under high humidity conditions at 28°C. Plated and high humidity treated tissue was examined under a binocular microscope after 4 days for evidence of spore production and subsequent identification of the pathogen by spore characteristics. Viability of spores was determined by monoconidial transfer to slopes of PDA.

Results and Discussion.

The results of seed germination, counts of the number of seedlings showing lesioning and the number of cotyledons from which the pathogen was positively identified by spore characteristics and colony growth are recorded in Tables 52 and 53.

TABLE 52. EFFECT of SURFACE SEED DRESSINGS on SEED GERMINATION and DISEASE INCIDENCE IN SEEDLINGS ARISING FROM DUSTED SEED.

GERMINATION.	Germination percentage.	
	7 days after Nursery Sowing.	Folded towel method 7 days.
Untreated	76	82
Fernasan dusted	71	76
Ceresan dusted	70	78

Germination figures calculated from counts of seedlings in the nursery are based on 1 oz. = 2,500 seeds.

SEEDLING NUMBERS.

	No. emerged 7 days.	No. transplanted 9 days.	Transplanting loss %
Untreated	1900	1,680	11.6
Fernasan dusted	1,780	1,600	10.1
Ceresan dusted	1,750	1,600	8.6

DISEASE INCIDENCE.

Days from Sowing	Number diseased seedlings.			Percentage diseased seedlings.		
	Non-dusted.	Fernasan dusted.	Ceresan dusted.	Non-dusted.	Fernasan dusted.	Ceresan dusted.
6	22	12	16	1.2	0.7	0.9
8	34	24	22	1.8	1.4	1.2
9	Transplanted.					
10	29	18	16	1.7	1.1	1.0
15	62	30	28	3.7	1.9	1.8
17	71	34	34	4.2	2.1	2.1
24	192	84	104	11.4	5.3	6.5
31	371	206	248	22.1	12.9	15.5
34	527	330	380	31.4	20.6	23.8
38	861	496	592	51.3	31.0	37.0
41	1,071	734	784	63.7	45.6	49.0

Reduction in number of diseased seedlings by seed dusting:-
Fernasan - 28.4%. Ceresan 23.1%.

TABLE 53. RECOVERY of A. ZINNIAE from LESIONED TISSUE ARISING FROM UNTREATED and DUSTED SEED.

Treatment.	% A. Zinniae from lesions plated to agar.	% A. Zinniae from lesions held under high humidity.
Untreated seed	72	61
Fernasan dusted seed	67	63
Ceresan dusted seed	63	57

The results of this nursery trial indicated that dusting zinnia seed with either Fernasan or Ceresan caused no appreciable depression in seed germination capacity or the number of seedlings which developed into saleable plants. This indicated that the use of these chemicals as surface seed dressings did not cause any great increase in the production of abnormal seedlings or retard the germination process.

The use of the chemical dusts used in this trial on a seedline which was carrying both surface and internal inoculum of A. zinniae, appeared to have little effect in reducing the number of diseased seedlings which developed.

There appeared to be very little difference in the relative effectiveness of the two chemicals used. Although the dusting of seed was noted as being extremely effective in reducing the number of fungi, in plating trials, which are considered to be surface borne and saprophytic on zinnia seed (e.g. A. tenuis and Mucor spp), these dusts were ineffective in controlling internal inoculum of A. zinniae associated with the seed.

In all cases the percentage recovery of A. zinniae from lesioned leaves, whether by plating or high humidity treatment, was high. Conidia from such lesions were proved viable on PDA.

The results of this trial support the claims (Neergaard 1945, Kispatic 1951, De Tempe 1959) for incomplete control of seedborne inoculum of A. zinniae associated with zinnia seed. On the other hand, they do not agree with the findings of

Beaumont et al. (1958) and it is suggested that the success obtained by these workers in controlling A. zinniae by surface seed dressings may have been due to th use of lines of seed which were carrying surface conidia of the pathogen but were not infected with A. zinniae. It would not seem likely that such outstanding success could have been obtained by Beaumont et al. (loc. cit.) by dusting the seed surface if the seedlines used had contained mycelium of the pathogen established within the tissues of the seed.

An approximate reduction of 25% in the number of diseased seedlings was recorded in seedlings from dusted seed compared to undusted seed. This reduction is thought to be due to the lethal effect of the chemical dusts on conidia of A. zinniae adhering to the seed surface. Accordingly, in seedlines which are not infected with the pathogen, but only contaminated with surface borne spores, dustin could be extremely effective as a control.

Since it has been shown that the majority of lines of zinnia seed imported to New Zealand carry internal as well as surface inoculum of A. zinniae, it is considered that the use of surface seed dressings is not effective in the complete control of seedborne inoculum of the pathogen.

STEEP TREATMENTS.

Seed treatments in which the chemical is applied by soaking seeds in a solution of the fungicide for a prescribed time have been widely used in controlling seedborne pathogens. By the use of this technique, in the present study, it was hoped that the fungicide would penetrate into the seed and prove lethal to inoculum of A. zinniae within the seed tissues.

Of those chemicals which have been used in the control of various seedborne diseases, mercuric chloride and sodium hypochlorite were chosen for investigation.

(a) Mercuric Chloride.

The fungicidal activity of mercuric chloride in weak solution is well known in the field of plant diseases, where it has long been used as a general purpose

surface sterilant of plant tissue. It has been used also in the control of certain fungal and bacterial diseases associated with the seed of various plants, e.g.,

Dwarf bunt (Tilletia brevifaciens Fischer) of wheat (E.F.P.C. 1954)

Fusarium and Verticillium spp in seed of China Aster (Callistephis chinensis) (Gloyer 1931)

Bacterial canker (Corynebacterium michiganense (Smith) Jensen) in tomato seed. (E.F.P.C 1954).

The use of mercuric chloride as a seed steep for control of A. zinniae has also been reported by various workers with varying success (Forsberg 1946, Schmidt 1953, Beaumont et al.1958).

On this basis it was decided to study the effect of immersing naturally diseased zinnia seed in a solution of 0.1% mercuric chloride for varying periods of time to determine whether this chemical was effective in controlling A. zinniae within the seed. As a corollary experiment the effect of the chemical on seed germination capacity was also investigated.

The seed used in this experiment was a naturally diseased line of variety Giant Dahlia Flowered. From seed sowing trials this seedline was shown to be diseased to the extent of 18%, 5% of inoculum being borne with the seed as internal mycelium and the remaining 13% as loose conidia on the seed surface (method page 181).

To determine what proportion of this 5% internal inoculum was associated with the tissues of the testa and how much was deep within the embryo of the seed, dissection and plating trials were conducted. From 200 manually dissected seeds the testas and embryos were washed in running tap water for 4 hours and plated separately to prune agar. By identification of the number of colonies of A.zinniae arising from plated tissue, which had been incubated at 28°C for 3 days, it was shown that of the 5% seed infection occurring in this seedline approximately 3% of the inoculum was present within the testa tissues and 2% within the embryo. Thus the analysis of the relative importance of inoculum associated with seed of this line was:-

Surface inoculum		13%
Internal inoculum tests	3%	
embryo	2%	5%
Total disease incidence	-	18%

Materials and Methods.

Seed samples of 5 gm (approximately 450 seeds) were used for each treatment. Each sample was placed on a 5" square of muslin and the corners drawn together to form a bag. Each was secured with a rubber band, ensuring that the seed was loose within the bags to allow quick penetration of the chemical. The seed samples were immersed in a 0.1% solution of mercuric chloride for various periods of treatment ranging from ½ minute to 6 hours. Following each period of immersion the sample was placed under running tap water for 10 minutes to prevent any further action by the $HgCl_2$ and the seed spread out on sterile blotting paper to dry at room temperature for 48 hours. During this drying process the seed was covered with another sheet of autoclaved blotting paper to reduce the risk of aerial contamination.

The dried seed from each treatment was tested for disease incidence by plating to prune agar (10 seeds per plate). The plates were incubated at 28°C for 5 days. Fungal colonies arising from the seed were cut out with a 5mm corkborer and the agar circles incubated inside a petri dish lid at room temperature for 48 hours. Identification was made by reference to spore morphology. Conidia of *A. zinniae* arising from the incubated agar circles were proved viable by transferring single spores to slopes of PDA. Untreated seed was also plated to prune agar to determine disease incidence.

Germination tests of untreated seed and of seed from each treated sample were made by the folded towel method at 25°C, results being recorded at 7 days.

Results and Discussion.

Results of disease incidence of *A. zinniae* arising from treated seed plated to agar and of the effects of various immersion times on seed germination are presented in Appendix VII and graphically in Figure 16.

These results show that there is a rapid initial decrease in disease incidence for periods of immersion up to 2 minutes, a drop from 10.5% to 5.5% being recorded. At periods of soaking from 2 to 14 minutes no reduction in disease incidence was shown. From 15 to 24 minutes immersion time a further reduction in disease

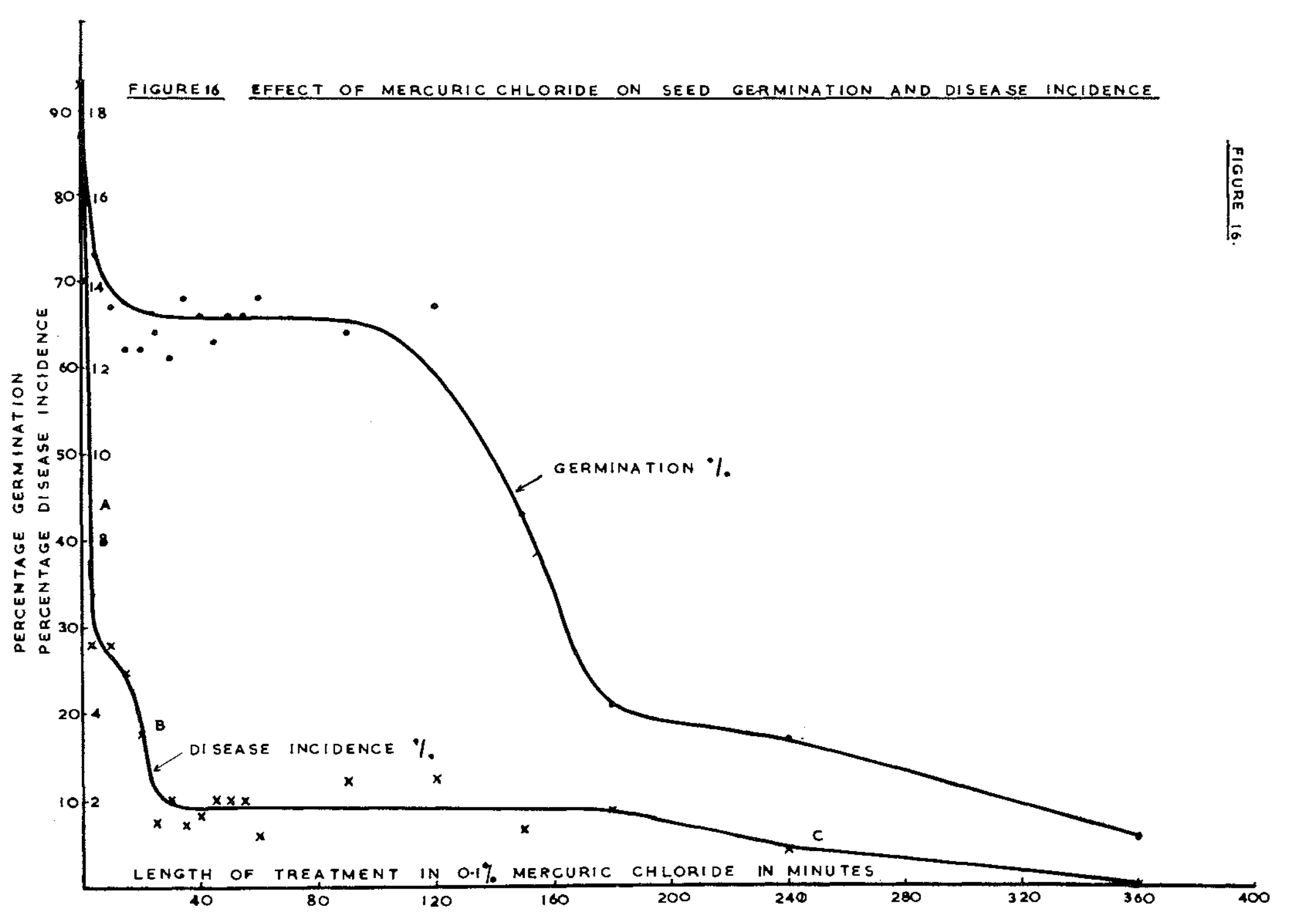


FIGURE 16 EFFECT OF MERCURIC CHLORIDE ON SEED GERMINATION AND DISEASE INCIDENCE
PERCENTAGE GERMINATION
PERCENTAGE DISEASE INCIDENCE
GERMINATION %
DISEASE INCIDENCE %
A
B
C
LENGTH OF TREATMENT IN 0·1% MERCURIC CHLORIDE IN MINUTES
90
80
70
60
50
40
30
20
10
18
16
14
12
10
8
4
2
40
80
120
160
200
240
280
320
360
400

FIGURE 16.

incidence from 5.0% to 2.0% was recorded. From this period of immersion (24 mins.) no further disease reduction with increased time of immersion was recorded until an immersion period of 180 minutes. After 360 minutes treatment the pathogen could no be recovered from treated seed plated to agar even when a repeat test was carried out.

Thus there were 3 immersion periods showing maximum effectiveness in reducing disease incidence of A. zinniae associated with zinnia seed (Figure 16):

1. A maximum drop after 2 minutes (A)
2. A progressive decline at periods from 14 to 24 minutes (B).
3. A final reduction at soaking periods from 180 to 360 minutes (C).

These three "critical"ranges of immersion period are explained by the followin

1. The maximum reduction at 2 minutes immersion from 18.5% to 5.5% corresponds to the amount of inoculum calculated previously as being present as loose conidia carried on the seedcoat. Thus soaking seed for 2 minutes in 0.1% mercuric chloride has proved lethal to surface borne inoculum.
2. The progressive decline in disease incidence at periods of immersion ranging from 14 to 24 minutes possibly represents the death of the fungal mycelium within the testa tissues, the longer treatment being required to kill inoculum deep within the testa.
3. The final drop in recovery of the pathogen from seed plated to agar occurs at treatment periods ranging from 180 to 360 minutes and is taken to indicate the lethal effect of mercuric chloride on myoelium of A. zinniae present in the tissues of the embryo.

Tests on seed germination capacity following increased periods of immersion showed an initial drop in viability from 87% in untreated seed to 67% in seed after 10 minutes immersion. From this point on however, germination capacity remained remarkably constant with increased seed immersion time up to 120 minutes. Beyond this time germination fell rapidly to very low figures at a period of immersion of 360 minutes.

Conclusions.

From the results of this experiment it was concluded that soaking seed in 0.1% mercuric chloride for periods up to 180 minutes was not effective as an absolute control of A. zinniae internally borne in zinnia seed. At 180 minutes treatment seed germination was markedly reduced. At the immersion period of 360 minutes required for absolute control of the pathogen, seed germination figures were reduced to non-practical levels.

It appeared that long immersion periods were necessary to allow penetration of the chemical into the seed, and that mercuric chloride had a more adverse effect on seed viability than it did on fungal mycelium within the embryo.

From the results in this study it appears that in seedlines carrying surface and testa borne inoculum only, mercuric chloride treatment at strength 1 in 1,000 for 25 minutes would prove adequate for control of the pathogen without adversely affecting seed germination.

The differential in immersion time adversely affecting seed germination and fungal viability precludes the use of mercuric chloride in controlling inoculum deep within the embryo. Hence this method of seed treatment cannot be recommended as a practical means of control of A. zinniae internally borne in zinnia seed.

SODIUM HYPOCHLORITE.

Studies were carried out to ascertain the effectiveness of immersing natural diseased zinnia seed in a solution of sodium hypochlorite for varying periods of time in the control of both externally and internally borne inoculum of A. zinniae

Materials and Methods.

The seedline used was a line of variety Giant Cactus Flowered which from sowing trials had been shown to give rise to approximately 17% of diseased seedlings. This seedline was also shown to be carrying both surface and deep-seated inoculum of A. zinniae to the extent of approximately 9% and 8% respectively.

The sodium hypochlorite solution used had been shown by titration with sodium thiosulphate to have an available chlorine content of 5.2%.

Seeds were placed in lots of 450 on squares of muslin, the corners being gathered and secured with a rubber band to form a bag. Each bag was immersed in the sodium hypochlorite solution for the prescribed period and then washed in running tap water for 10 minutes. The seeds were removed from the muslin and spread out to dry between two sheets of sterile blotting-paper for 1 hour. Two hundred seeds were germinated by the folded towel method at 25°C. The remainder were plated directly to prune agar and the plates held at 28°C for 4 day

Germination percentages were recorded at 7 days.

Fungal colonies arising from plated seed were cut with a corkborer, the agar circles allowed to incubate at 28°C for 48 hours, and positive identification of the pathogen made by reference to spore characteristics.

Results and Discussion.

Results of seed germination capacity following various periods of immersion in sodium hypochlorite, and the percentage of A. zinniae identified from treated seed plated to agar are recorded in Appendix VIII and graphically in Figure 17.

These results indicate that immersion of seed in sodium hypochlorite solution for up to 6 hours reduced seedborne inoculum to very low levels but did not

FIGURE 17. EFFECT OF SODIUM HYPOCHLORIDE IMMERSION ON SEED GERMINATION CAPACITY AND DISEASE INCIDENCE.

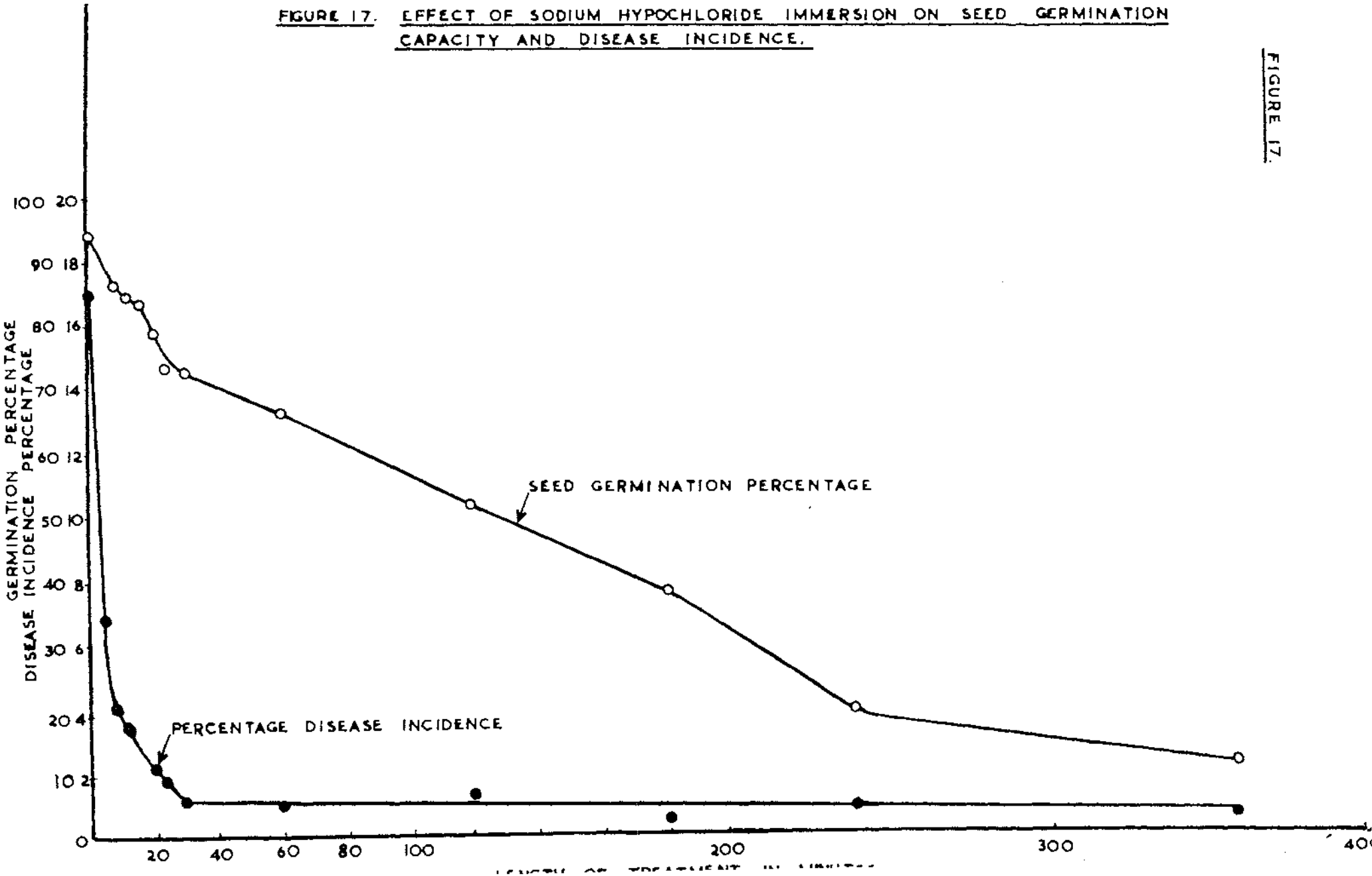


FIGURE 17.

completely eliminate the pathogen from the seed. At periods of immersion longer than 2 hours, seed germination capacity was greatly impaired. These results indicate that sodium hypochlorite immersion periods of long duration are required to kill inoculum of A. zinniae within zinnia seed; which periods are extremely detrimental to seed germination capacity.

Conclusions.

The results of this study on the effectiveness of sodium hypochlorite as a seed soak showed that the use of this chemical reduced disease incidence to low levels (0.5 - 1.0%). However the effect of the sodium hypochlorite on the germination capacity of the seed was very severe at periods of immersion likely to be effective in controlling the pathogen. As a result it is concluded that it would be impractical to use this method as the basis of a recommendation for contr of internally borne inoculum of A. zinniae associated with zinnia seed.

ANTIBIOTICS.

In recent years increasing attention has been paid to the possibility of using antibiotics for the control of plant diseases. Apart from a fungicidal or bacteriocidal effect, several antibiotics possess the property of penetrating into seed tissues. This offers the possibility of their usefulness in combating internal pathogens in seed.

One notable instance of the use of antibiotics has been the success obtained by Dekker (1957) in the control of Ascochyta pisi internally borne in pea seed, by the use of seed soaks containing the antibiotics Rimocidin and Pimaricin (produced by the micro-organisms Streptomyces rimosus and S. natalensis respectively).

Dekker (1955) states that "so far about 100 antibiotics have been found that are toxic to plant parasites. Only one antibiotic, however, has been adopted in practice, viz. streptomycin, which, with or without the addition of some terramyci

is used against fireblight, a bacterial disease of pear and apple trees in the U.S.A."

In the present study attempts were made to determine whether the antibiotic streptomycin (produced by Streptomyces griseus) could be used to control internally borne mycelium of A. zinniae in zinnia seed.

Streptomycin sulphate powder (740 mgm/gm) was prepared into a stock solution of 4,000 ppm (100 mgm streptomycin sulphate + 18.5 ml. distilled water) and this stock solution was diluted to give a range of concentrations from 4,000 ppm. down to 1 ppm. Each solution was incorporated into PDA and the effect of different strengths of streptomycin on colony growth of A. zinniae at 28°C recorded. There was a reduction of growth even at 1 ppm, with complete inhibition of growth at 2,000 ppm. (Table 54).

TABLE 54. EFFECT of STREPTOMYCIN SULPHATE in PDA on COLONY DIAMETER of A. ZINNIAE.

Conc. Streptomycin Sulphate. ppm.	Colony Diameter A. zinniae 10 days, 28°C (mm).
0	84
1	77
5	72
10	69
25	66
50	63
75	60
100	58
120	56
200	52
500	39
750	26
1,000	9
2,000	0
4,000	0

In order to determine the effectiveness of streptomycin in controlling A. zinniae carried as mycelium in zinnia seed, further studies were carried out.

A line of variety Lilliput which had previously been shown to be both contaminated and infected with the pathogen to the extent of 7% and 12% respectively, was tested. Seed was placed in solutions of streptomycin sulphate (50gm.seed per 100 ml solution) at concentrations from 50 ppm to 4,000 ppm and held at 28°c

for 24 hours.

Germination of each seed sample following soaking was assessed by the folded towel method at 25°C.

Two methods were used for the assessment of the effect of the antibiotic on disease incidence. In the first method the seeds were placed on moist filter paper in a flat tray covered by a glass plate, and a count made of the number of seeds from which mycelium grew. This method was called the filter paper test. In the second method the seeds were removed from the streptomycin solution, washed in sterile water and plated directly to prune agar. This method was called the plating method.

Results are presented in Table 55.

TABLE 55. EFFECT of STREPTOMYCIN SOLUTIONS on A. ZINNIAE ASSOCIATED WITH ZINNIA SEED.

Strept. conc. ppm.	Germ'n. % 7 days.	Filter Paper Test (200 seeds). No. seeds yielding A. zinniae.	% A.zinniae.	Plating Method (150 seeds). No. seeds yielding A. zinniae.	% A.zinniae.
0	86	34	17.0	30	20.0
50	83	20	10.0	21	14.0
75	80	24	12.0	20	13.3
100	81	24	12.0	19	12.7
135	78	20	10.0	20	13.3
200	78	24	12.0	18	12.0
300	76	23	11.5	19	12.7
500	76	19	9.5	17	11.3
760	76	14	7.0	18	12.0
1,000	73	13	6.5	14	9.3
1,320	68	11	5.5	16	10.7
2,000	64	13	6.5	11	7.3
4,000	46	11	5.5	14	9.3

Although these results indicated that streptomycin sulphate had some disinfecting action, the pathogen was not eradicated from the seed. Increasing the concentration of antibiotic in the soak solution did not appear to have any appreciable effect, while at high concentrations there was a risk of retarding the germination.

Attempts to increase the penetration of the antibiotic by adding a wetting agent or glycerine to the solution were unsuccessful. Lengthening the duration of the soaking period to 48 hours also did not appear to reduce disease incidence, but seed germination was seriously impaired.

From these studies on the effect of streptomycin as a disinfectant of zinnia seed it is concluded that, although soaking seed in solutions containing up to 4,000 ppm streptomycin sulphate allowed some reduction in the percentage of A. zinniae associated with zinnia seed, the benefits obtained from the use of this material were more than outweighed by the effect of the antibiotic in reducing seed germination capacity. Accordingly the use of streptomycin solutions as a seed soak is concluded to be of no practical use in the control of A. zinniae in zinnia seed lines infected with the pathogen.

3. SUMMARY and CONCLUSIONS.

Several methods were studied to try to control *A. zinniae* by means of seed treatment.

The hot-water treatment method which had been advocated as being effective overseas (Baker 1956) was found to be very severe on seed germination and to be ineffective in completely eliminating seedborne inoculum of the pathogen under New Zealand conditions. The more marked depression in seed germination capacity under New Zealand conditions than those recorded overseas was concluded to be due, at least in part, to the fact that all seed sown commercially in New Zealand is at least one year old from harvest, and is more readily damaged by hot water than is freshly harvested seed.

The water-soak method and the use of mercuric chloride and sodium hypochlorite steep treatments were found to reduce disease incidence of *A. zinniae* to low levels (0.5 - 1.5%) but also caused depression in seed viability.

The use of surface seed dressings and the use of antibiotics as seed soaks were both concluded to be ineffective in controlling internal inoculum of the pathogen.

In all the methods studied to try to control *A. zinniae* by means of seed treatment, the germination of the seed was more adversely affected than inoculum of the pathogen within the seed embryo. The majority of the methods studied reduced disease incidence to low level without seriously impairing the germination of the seed, but none of the seed treatments completely eliminated *A. zinniae* without this severe germination reduction.

Thus it was concluded that the seed embryo of zinnia was in all cases more susceptible to the treatment used than was mycelium of the pathogen within the embryo tissues. This would indicate that none of the methods studied could be effective in eliminating *A. zinniae* within the seed and yet allow the retention of even a moderate (30 - 50%) seed germination figure.

It should be emphasised however that although no control method was found

which would eliminate A. zinniae from zinnia seed without seriously affecting seed germination capacity, the hot-water treatment method, the water-soak method, and the mercuric chloride and sodium hypochlorite steep treatments greatly reduced disease incidence in seedlings arising from treated seed. In this respect these methods could be of use in reducing the rate of buildup of the disease in commercial plantings. Accordingly, these treatments could allow the production of more saleable plants in commercial nurseries than would be obtained from plantings of untreated seed.

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APPENDIX I

COMPOSITION AND PREPARATION OF MEDIA

1. ARTIFICIAL MEDIA

(a) Laboratory Potato Dextrose Agar

Agar	12 g.
Peeled sliced potatoes	200 g.
Dextrose	10 g.
Water (distilled)	1,000 ml.

Preparation: The potatoes were boiled gently for an hour in 500 ml. of water, after which the liquid was separated by straining through cheesecloth. Agar was melted in 500 ml. of water and added to the filtrate. Dextrose was added and the total volume adjusted to 1,000 ml. Approximate pH 5.8.

Sterilisation: Unless otherwise stated all media were sterilised in an autoclave at 15 p.s.i. for 20 mins. immediately after preparation.

(b) Potato Dextrose Agar (Difco or Oxoid)

Potato infuxion	200 g.
Bacto Dextrose	20 g.
Bacto Agar	15 g.

Preparation: 39 grams of the above mixture were suspended in 1,000 ml. of distilled water and boiled to dissolve the medium. Approximate pH 5.7.

(c) Cornmeal Agar

Agar	17 g.
Cornmeal	20 g.
Water (distilled)	1,000 ml.

Preparation: As for 1(a). Approximate pH 5.4.

(d) Czapek-Dox Agar

Agar	15.00 g.
$NaNO_3$	3.00 g.
K_2PO_4	1.00 g.
$MgSO_4.7H_2O$	0.50 g.
KCl	0.50 g.
$FeSO_4.7H_2O$	0.01 g.
Sucrose	30.00 g.
Water (distilled)	1,000 ml.

Preparation: The nutrients were dissolved in 500 ml. of hot water and mixed with the agar which had been dissolved in 500 ml. of water. Approximate pH 6.6.

(e) Nutrient Agar.

Oxoid nutrient agar	28 g.
Water (distilled)	1,000 ml.

Preparation: The above were boiled in a steam bath to dissolve the agar. Approximate pH 6.7.

(f) Sabourand Agar (Maltose)

Sabourand Agar	70 g.
Water (distilled)	1,000 ml.

Preparation: As for 1(e). Approximate pH 5.5.

(g) Tomato Juice Agar

Oxoid tomato juice agar	52 g.
Water (distilled)	1,000 ml.

Preparation: As for 1(e). Approximate pH 5.6.

(h) V8 Juice Agar.

V8 juice (commercial)	150 ml.
Agar	16 g.
Water (distilled)	850 ml.

Preparation: as for 1(e). Approximate pH 4.3.

(i) Malt Agar

Malt extract (Difco)	20 g.
Agar	25 g.
Water (distilled)	1,000 ml.

Preparation: As for 1(e). Approximate pH 5.9.

(j) Dextrose Agar.

Agar	15 g.
Beef extract	3 g.
Tryptose	10 g.
Dextrose	10 g.
Nace	5 g.
Water (distilled)	1,000 ml.

Preparation: As for 1(e). Approximate pH 6.8.

(k) Prune Agar.

Prune infusion	36 g.
Bacto-agar	15 g.

Preparation: 24 grams of this mixture were suspended in 1,000 ml. distilled water and boiled to dissolve the medium. Approximate pH 5.5.

(l) Water Agar.

Agar	12 gm.
Water (distilled)	1,000 ml.

Preparation: Agar dissolved in hot water. Approximate pH 4.7.

(m) Oatmeal Agar

Oatmeal	20 gm.
Agar	12 g.

Water (distilled)	1,000 ml.

Preparation: As for 1(a). Approximate pH 6.3.

(n) Mycological Peptone Agar. (Oxoid)

Mycological peptone preparation	50 g.
Agar	15 g.
Water (distilled)	1,000 ml.

Preparation: As for 1(e). Approximate pH 5.6.

(o) Tryptone-Glucose-Yeast Extract Agar (TYGE)

Yeast extract	2.5 g.
Tryptone	5 g.
Glucose	1 g.
Agar	12.5 g.
Water (distilled)	1,000 ml.

Preparation: As for 1(e). Approximate pH 6.2.

(p) Glucose-Lactose-Phosphate Agar (GLPA)

Beef extract	2 g.
Lactose	4 g.
Glucose	0.4 g.
Peptone	1.6 g.
$Na_2HPO_4.12H_2O$	1.2 g.
Water (distilled)	1,000 ml.

Preparation: As for 1(e). Approximate pH 6.1.

(q) Soil Agar

Autoclaved loam soil	40 g.
Agar	4 g.
Water (distilled)	1,000 ml.

Preparation: As for 1(a). Approximate pH 6.2.

2. NATURAL MEDIA

Method of preparation as on page 18.

3. PLANT EXTRACT MEDIA

Method of preparation as on page 18.

(a) Zinnia blossom Agar.

Zinnia blossom	450 g.
Agar	12 g.
Water (distilled)	1,000 ml.

Approximate pH 6.6.

(b) Zinnia leaf Agar.

Zinnia leaves	450 g.
Agar	12 g.
Water (distilled)	1,000 ml.

Approximate pH 6.9.

(c) Zinnia seed Agar.

Zinnia seed	28 g.
Agar	12 g.
Water (distilled)	1,000 ml.

Approximate pH 6.7.

(d) Tobacco leaf Agar.

Tobacco leaves	450 g.
Agar	12 g.
Water (distilled)	1,000 ml.

Approximate pH 6.4.

(e) Marigold leaf Agar.

Marigold leaves	450 g.
Agar	12 g.
Water (distilled)	1,000 ml.

Approximate pH 6.1.

(f) Tomato leaf Agar.

Tomato leaves	450 g.
Agar	12 g.
Water (distilled)	1,000 ml.

Approximate pH 5.7.

(g) Lettuce leaf Agar.

Lettuce leaves	450 g.
Agar	12 g.
Water (distilled)	1,000 ml.

Approximate pH 6.5.

APPENDIX II

RANGE OF DIMENSIONS OF CONIDIA PRODUCED ON DIFFERENT MEDIA

1. ARTIFICIAL MEDIA

Medium	Conidial Dimensions in microns			
	Total length	Body Length	Beak length	Body width
Lab. PDA	63.6-174.3	24.6-57.4	34.9-116.9	16.4-24.6
Oxoid PDA	90.2-215.3	36.9-61.5	51.3-153.8	12.3-20.5
Difco PDA	106.6-229.6	36.9-77.9	65.6-151.7	16.4-24.6
Prune Agar		26.6-65.6		
Water agar	147.6-278.8	47.2-73.8	98.4-229.6	14.4-24.6
Nutrient agar	no sporulation.			
Czapek-Dox agar	75.9-168.1	41.0-65.6	28.7-102.5	14.4-18.5
Mycol.peptone agar	71.8-184.5	32.8-65.6	36.9-139.4	16.4-22.6
Sabourand agar	45.1-127.1	28.7-55.4	10.3-77.9	16.4-28.7
Cornmeal agar	123.0-229.6	55.4-69.7	63.6-164.0	14.4-41.0
Soil agar	116.9-305.5	49.2-61.5	55.4-250.1	12.3-18.5
Malt agar	114.8-145.6	45.1-75.9	35.4-86.1	14.4-28.7
Oatmeal agar	133.0-258.3	55.4-75.9	73.8-188.6	16.4-26.7
Tomato juice agar	39.0-143.5	20.5-63.9	16.4-82.0	16.4-26.7
TYGE agar	71.8-139.4	26.7-45.1	43.1-98.4	14.4-32.8
V8 juice agar	69.7-106.6	34.9-57.4	12.3-65.6	16.4-28.7
Dextrose agar	no sporulation.			
G.L.P. agar	no sporulation.			

2. NATURAL MEDIA

Zinnia blossom agar	82.0-227.5	45.1-82.0	36.9-145.5	16.4-30.7
Zinnia leaf agar	84.0-246.0	41.0-65.6	41.0-194.7	12.3-24.6
Zinnia seed agar	57.4-143.5	18.4-57.4	16.4-92.3	6.2-20.5
Tobacco leaf agar	51.3-205.0	30.8-61.5	20.5-151.7	10.3-20.5
Tomato leaf agar	53.3-225.5	31.8-61.5	18.5-157.9	8.2-16.4
Lettuce leaf agar	43.1-157.9	34.9-61.5	8.2-96.4	10.2-24.6

3. PLANT EXTRACT MEDIA

Zinnia blossom agar	63.6-151.7	30.8-51.3	32.8-96.4	10.3-16.4
Zinnia leaf agar	57.4-139.4	28.7-51.3	22.6-106.6	8.2-12.3
Zinnia seed agar	61.5-205.0	32.8-55.4	28.7-161.9	8.2-14.4
Tobacco leaf agar	no sporulation.			
Marigold leaf agar	55.4-135.3	28.7-47.2	18.5-96.4	8.2-14.4
Tomato leaf agar	77.9-231.7	22.6-55.4	36.9-176.3	10.3-18.5
Lettuce leaf agar	59.5-149.6	30.8-45.1	28.7-102.5	8.2-14.4

APPENDIX III

DISTRIBUTION OF CONIDIAL DIMENSIONS WITHIN DIFFERENT ISOLATES

A. BODY LENGTH

Range in microns	Origin of Isolate					
	Root	Stem	Cotyledon	Seed coat	Adult Leaf	Blossom
20-30	-	-	-	-	-	-
31-40	2	2	-	2	2	11
41-50	23	12	-	17	11	21
51-60	20	26	17	16	23	14
61-70	5	10	24	14	11	4
71-80	-	-	9	1	2	-
81-90	-	-	-	-	1	-

B. TOTAL LENGTH

Range in microns	Origin of Isolate					
	Root	Stem	Cotyledon	Seed coat	Adult Leaf	Blossom
80-100	-	-	-	-	-	-
101-120	-	2	2	-	-	-
121-140	-	6	2	-	-	-
141-160	1	6	5	-	2	-
161-180	6	5	7	1	2	2
181-200	7	10	14	2	2	3
201-220	19	14	10	3	6	3
221-240	11	5	5	3	9	12
241-260	6	1	4	14	17	18
261-280	-	1	1	8	3	8
281-300	-	-	-	7	2	3
301-320	-	-	-	5	2	1
321-340	-	-	-	4	3	-
341-360	-	-	-	3	2	-
361-380	-	-	-	-	-	-

APPENDIX IV

PERCENTAGE GERMINATION FOR SPORES OF A. ZINNIAE HELD AT DIFFERENT TEMPERATURES

CUMULATIVE HOURS	TEMPERATURE °C.														
	-5	41	5	10	17	20	22	24	26	28	30	33	35	37	40
0.5								0.3	1	4	2				
1.0							7	11	14	21	20	5			
1.5					2	5	32	40	48	51	46	17	6		
2.0				1	26	37	44	52	56	63	59	37	19	5	
2.5		1	4	4	44	50	54	64	66	81	73	59	27	31	
3.0		2	4	5	53	58	63	71	72	86	83	69	36	37	2
3.5		4	11	29	60	64	72	76	77	88	84	75	49	40	7
4.0		5	12	41	71	75	79	81	86	89	89	79	NR	46	9
4.5		6	27	48	70	76	73	81	87	90	89	81	NR	48	NR
5.0		7	42	52	80	77	75	86	90	94	91	84	69	56	12
5.5		7	44	58	81	82	82	89	90	97	94	90	78	NR	21
6.0		6	61	61	82	83	87	90	96		96	92	78	NR	26
6.5		7	69	NR	85	84	NR	92				94	81	NR	NR
7.0		5	71	68	88	89	89	96				95	85	66	NR
7.5		7	73	70	91	93	92						88	70	32
8.0		8	66	77	94	97	95						91	77	36
9.5		NR	72	81	97								97	81	47
10.0		NR	72	83										83	52
10.5		NR	75	87										86	NR
11.0		NR	77	89										90	NR
12.0		NR	81	89										92	71
13.0		NR	84	90										96	74
14.0		NR	80	96											NR
17.0		15	87												77
18.0		NR	92												NR
20.0		NR	96												NR
24.0		NR													89
25.0		27													
36.0		31													
60.0		42													
85.0		46													
183.0		73													
204.0		80													

Germination criterion - when length of germ tube equalled spore body width.

Each figure represents an average of counts of 3 x 100 spores to the nearest whole number.

NR = Not recorded.

APPENDIX V.

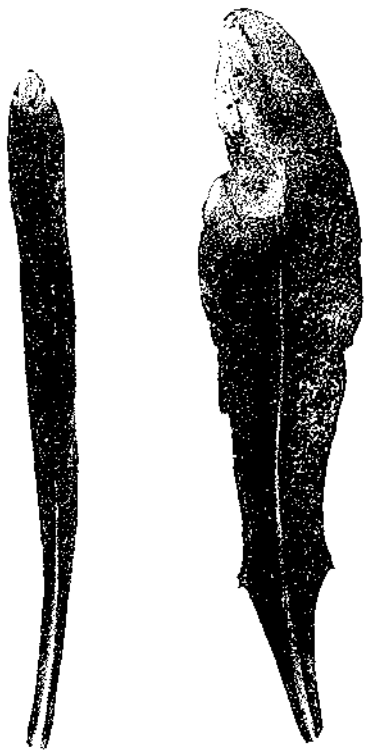
SYMPTOM EXPRESSION OF A. ZINNIAE ON DIFFERENT HOST SPECIES.

Artificially inoculated Black Nightshade (Solanum nigrum L.)

Artificially inoculated Lettuce (Lactuca sativa L.)

Artificially inoculated Cineraria (Senecio cruentus L.)

Artificially inoculated Tomato (Lycopersicum esculentum Mill)



Artificially inoculated Cornflower (*Centaurea cyanus* L.)

APPENDIX VI

SEED SCREENING TRIALS - DISEASE INCIDENCE IN

IMPORTED SEEDLINES 1962,1963

A. 1962	Percentage Diseased Seedlings	
Variety	Sowing method at 10 days	Blotter method at 7 days
Californian Giants	5.0	7.0
Giant Dahlia flowered	0.5	1.5
Fumila elegans dwarf	13.0	12.0
Super Giants	21.5	23.0
Fumila elegans	6.0	7.0
Giant Dahlia flowered	4.0	5.5
Lilliput	7.0	9.0
Giant Dahlia Flowered	3.0	3.5
Peppermint Stick	2.5	5.5
Persian Carpet	4.0	4.0
Californian Giants	5.5	3.5
Fumila elegans	1.0	3.0
Fiesta Lights	6.5	9.5
Giant Cactus Flowered Burpee	2.5	7.0
Persian Carpet	3.0	3.5
Orthopolka	11.0	10.5
Giant Cactus Flowered Burpee	0.5	4.5
Giant Cactus Flowered (Pride of Dieldrin)	2.0	4.0
Giant Dahlia Flowered	5.5	5.0
Lilliput	5.5	6.5
Giant Dahlia Flowered (Cherrytime Red)	6.5	9.0
Giant Cactus Flowered (Blaze)	3.5	4.5
Orthopolka	5.0	7.0
Giant Dahlia Flowered	2.5	4.0
Giant Cactus Flowered (Dark Jewels)	12.5	16.0
B. 1963		
Giant Dahlia Flowered	5.5	6.0
Giant Cactus Flowered	3.5	3.0
Orthopolka giant	2.5	3.5
Thumbelina	2.5	3.5
Giant Cactus Flowered (Dark Jewels)	4.5	6.5
Persian Carpet	2.0	3.5
Cupid	6.5	7.0
Elegans Fumila	3.0	4.0
Fantasy	5.0	5.5
California Giants	7.0	7.5
Lilliput	3.5	4.0
Giant Dahlia Flowered (Polar Bear)	4.5	4.0
Giant Dahlia Flowered (Oriole)	3.0	4.5
Giant Dahlia Flowered (Canary Bird)	2.5	3.0
Giant Dahlia Flowered (Exquisite)	2.5	6.5
Giant Dahlia Flowered (Eldorado)	3.0	4.0
Giant Dahlia Flowered (Dream)	3.0	2.5
Giant Dahlia Flowered (Golden State)	3.5	3.0
Giant Dahlia Flowered (Illumination)	5.0	7.0
Giant Dahlia Flowered (Golden Dawn)	7.5	7.0
Orthopolka	2.5	3.5
Giant Cactus Flowered (Blaze)	3.0	4.5
Linearis Orange	2.0	2.0
Lilliput (Pink Gem)	3.5	4.5

Lilliput (Canary Gem)	4.5	4.5
Lilliput (White Gem)	4.5	4.0
Persian Carpet	5.0	4.5
Giant Dahlia Flowered (Will Rodgers)	4.0	6.0
Lilliput (Crimson Gem)	5.0	7.5
Giant Cactus Flowered (Floradale Scarlet)	5.5	7.5
Giant Dahlia Flowered (Purple Prince)	6.0	4.5
Giant Dahlia Flowered (Royal Purple)	7.0	6.5
Cupid	3.5	5.5
Californian Giants	5.5	6.0
Giant Dahlia Flowered (Luminosa)	6.0	7.5
Giant Dahlia Flowered (Meteor)	5.5	7.5
Giant Dahlia Flowered (Crimson Monarch)	3.0	5.0
Giant Dahlia Flowered (Scarlet Flame)	8.0	11.0
Giant Cactus Flowered (Riverside Beauty)	3.0	6.5
Giant Dahlia Flowered	8.0	5.5
Elegans Pumila	5.0	7.0
Fantasy	11.5	10.5
Burpee Hybrids	4.5	4.5
Giant Cactus Flowered (Dark Jewels)	2.5	6.5
Lilliput (Black Ruby)	5.5	5.0
Haageana	2.5	3.0
Thumbelina	3.0	3.0
Lilliput (Purple Gem)	1.5	4.0
Lilliput	7.0	9.5
Lilliput pastels	6.0	7.5
Lilliput (Lilac Gem)	5.5	6.0
Harmony type Scabious	3.5	3.0
Lilliput (Scarlet Gem)	3.0	4.0
Lilliput (Golden Gem)	7.0	6.0
Lilliput (Salmon Gem)	3.5	6.5
Super Giants	8.5	6.0
Lilliput (Valencia)	2.5	4.0
Peppermint-stick	6.0	5.5
Giant Cactus Flowered (Cherrytime)	7.5	6.5
Lilliput (Rosebud)	6.0	6.5
Giant Dahlia Flowered (Crimson Monarch)	6.0	7.0
Giant Dahlia Flowered (Golden Dawn)	4.5	6.5
Giant Dahlia Flowered (Exquisite)	2.0	5.5
Giant Dahlia Flowered (Polar Bear)	3.0	3.5
Giant Dahlia Flowered (Canary Bird)	4.0	7.0
Giant Dahlia Flowered (Scarlet Flame)	3.5	4.0
Giant Dahlia Flowered (Oriole)	3.0	3.0
Giant Dahlia Flowered (Purple Prince)	6.5	7.5
Giant Dahlia Flowered (Illumination)	4.0	5.0
Lilliput	3.0	6.5
Burpee Hybrids	3.5	4.5
Giants of California	4.0	10.0
Pumila Elegans	3.5	4.0
Blaze	4.0	3.0
Thumbelina	2.0	5.5
Merry-Go-Round	9.0	9.5
Miss Universe	7.0	6.0
Orthopolka	4.0	4.0
Crown of Gold	4.0	6.5
Sunny-Boy	6.5	5.5
Scabious	5.0	4.0

Grand Canyon	4.5	8.0
Giant Dahlia Flowered	9.5	7.0
Giant Cactus Flowered (Burpee)	7.0	7.0
Scabious Flowered	6.0	8.0
Creeping Zinnia	0.0	1.5
Cupid	5.0	2.0
Red Riding Hood	1.0	3.0
Persian Carpet	2.0	3.0
Linearis Orange	1.5	2.5
Old Mexico	0.0	0.5
Firecracker Clear Red	3.0	4.5
Red Man	8.0	6.0
Perennial Snowflake	1.0	2.0
Persian Carpet	2.0	3.0
Dark Jewels	4.0	5.0
Lilliput Pompom	4.5	5.0
Cherrytime	3.5	5.0
Giant Cactus Flowered (Blaze)	1.0	3.0
Giant Dahlia Flowered	2.0	4.0

APPENDIX VII

EFFECT OF MERCURIC CHLORIDE IMMERSION ON SEED GERMINATION AND DISEASE INCIDENCE

IMMERSION TIME IN MINUTES	SEED GERMINATION PERCENTAGE 7 DAYS	DISEASE INCIDENCE - PLATED SEED			
		NO. SEEDS PLATED	NO. FUNGAL GROWTHS	NO. YIELDING A. ZINNIAE	% A. ZINNIAE
0.(untreated)	87	200	74	37	18.5
0.5	86	200	49	28	14.0
1	85	200	46	26	13.0
2	81	200	18	11	5.5
3	80	200	16	9	4.5
4	78	200	15	11	5.5
5	76	200	18	9	4.5
6	73	200	16	10	5.0
7	72	200	17	8	4.0
8	70	200	27	11	5.5
9	69	200	15	12	6.0
10	67	200	25	11	5.5
11	66	200	19	11	5.5
12	67	200	10	12	6.0
13	64	200	29	12	6.0
14	64	200	17	9	4.5
15	62	200	20	10	5.0
16	63	200	29	7	3.5
17	65	200	27	8	4.0
18	60	200	29	7	3.5
19	63	200	22	10	5.0
20	62	200	13	7	3.5
21	65	200	16	5	2.5
22	64	200	20	5	2.5
23	66	200	17	2	1.0
24	66	200	18	4	2.0
25	64	200	13	3	1.5
26	60	200	19	3	1.5
27	66	200	23	4	2.0
28	67	200	28	3	1.5
29	65	200	11	4	2.0
30	61	200	19	4	2.0
35	68	220	21	3	1.4
40	66	243	23	4	1.6
45	63	221	27	5	2.0
50	66	225	19	5	2.0
55	66	220	21	5	2.0
60	68	210	17	3	1.2
90	64	121	12	3	2.5
120	67	114	15	3	2.6
150	43	142	7	2	1.4
180	21	119	5	2	1.8
240	17	116	6	1	0.9
360	6	127	7	0	0.0
360(repeat)	8	200	9	0	0.0

APPENDIX VIII.

EFFECT OF SODIUM HYPOCHLORITE IMMERSION ON SEED GERMINATION CAPACITY AND DISEASE INCIDENCE.

IMMERSION TIME IN MINUTES.	SEED GERMINATION PERCENTAGE 7 DAYS.	DISEASE INCIDENCE - PLANTED SEED. NO. SEEDS PLANTED.	NO. YIELDING A. ZEAE.	% A. ZEAE.
0 (untreated)	94	250	42	16.8
0.5	92	243	28	11.5
1	89	234	21	9.0
2	88	253	19	7.5
4	90	248	16	6.5
6	88	250	13	5.2
8	86	242	10	4.1
10	84	227	9	4.0
15	85	131	5	3.8
20	78	185	4	2.2
25	73	164	3	1.8
30	72	180	2	1.1
60	66	200	2	1.0
120	52	200	3	1.5
180	38	200	1	0.5
240	21	200	2	1.0
360	10	200	1	0.5
360 (repeat)	12	200	1	0.5

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