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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
Master of Agricultural Science
at Massey University of Manawatu.

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by
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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 raised 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.

Alternaria 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 artifically.
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 glass-house 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 ¼" 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 traya 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".