STUDIES ON GENETIC AND ON CHEMICALLY INDUCED RESISTANCE OF CUCUMBER TISSUES TO CLADOSPORIUM CUCUMERINUM

M. K. EL-DIN FOUAD

THEOREMS

I

Varieties of one plant species may show different mechanisms of resistance to the same disease.

II

The presence of *Cladosporium cucumerinum* within the host tissues affects the pectin of the cell walls in such a way that these cells retain their reaction to ruthenium red after treatment with hot HCl.

III

If systemic fungicides come in general use they will be preferable to the standard surface fungicides which on spraying do not penetrate or penetrate only to a slight extent.

IV

Although anatomical investigations show that in both genetic and chemically induced resistance to cucumber scab, penetration of the host surface rarely occurs, yet the two types of resistance differ in the mechanism by which this resistance to penetration is brought about.

V

In Egyptian sweet melons, fruit setting is independent of the number of hermaphroditic flowers produced by the plant. This fact is against the recommendation once revealed that muskmelons should be "nipped" in order to get more yield.

VI

The fact that size and quality of fruits of the Egyptian sweet melons are badly affected during summer period, is probably caused by the harmful influence of high temperature on the balance between assimilation and dissimilation processes.

VII

On sowing in a clay soil in Egypt covering cotton seeds with sand may cause resistance to soreshin disease, hence early and high yield is attained.

VIII

The future of peach growing in Egypt largely depends on establishing bushy trees.

IX

The effect of the rootstock on the scion in woody plants can be predicted before grafting by investigating the anatomical structure of the rootstock.

X

The way of irrigation adopted by the Egyptian farmers nowadays is probably responsible for great losses in the yield of many crops.

XI

The spread of disease is an important cause of the relatively slow progress of nations in tropical and sub-tropical countries.

Diss. Fouad
Wageningen 1956

STUDIES ON GENETIC AND ON CHEMICALLY INDUCED RESISTANCE OF CUCUMBER TISSUES TO CLADOSPORIUM CUCUMERINUM (ELL. & ARTH.)

met een samenvatting

ONDERZOEKINGEN OVER GENETISCHE EN CHEMISCH GEINDUCEERDE RESISTENTIE VAN KOMKOMMER TEGEN CLADOSPORIUM CUCUMERINUM (ELL. & ARTH.)

Dit proefschrift met stellingen van

MOHAMED KAMAL EL-DIN FOUAD

landbouwkundig ingenieur, geboren te Cairo (Egypte), 16 februari 1924, is goedgekeurd door de promotor, Dr A. J. P. OORT, hoogleraar in de plantenziektenkunde.

De Rector Magnificus der Landbouwhogeschool,

J. H. BECKING

Wageningen, 9 mei 1956

STUDIES ON GENETIC AND ON CHEMICALLY INDUCED RESISTANCE OF CUCUMBER TISSUES TO CLADOSPORIUM CUCUMERINUM (ELL. & ARTH.)

MET EEN SAMENVATTING

ONDERZOEKINGEN OVER GENETISCHE EN CHEMISCH GEINDUCEERDE RESISTENTIE VAN KOMKOMMER TEGEN CLADOSPORIUM CUCUMERINUM (ELL. & ARTH.)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS DR. J. H. BECKING,
HOOGLERAAR IN DE HOUTMEETKUNDE, DE BOSBEDRIJFSECONOMIE,
DE BOSBEDRIJFSREGELING EN DE HOUTTEELT
EN BOSBESCHERMING IN DE TROPEN,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
DER LANDBOUWHOGESCHOOL TE WAGENINGEN
OP DONDERDAG 5 JULI 1956 TE 16 UUR

DOOR

MOHAMED KAMAL EL-DIN FOUAD

H. VEENMAN & ZONEN – WAGENINGEN – 1956

STUDIES ON GENETIC AND ON CHEMICALLY INDUCED RESISTANCE OF CUCUMBER TISSUES TO CLADOSPORIUM CUCUMERINUM (ELL. & ARTH.)

THESIS

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF
AGRARIAN SCIENCES
AT THE AGRICULTURAL UNIVERSITY OF WAGENINGEN
ON THURSDAY, 5 JULY 1956 AT 16 O'CLOCK

BY

MOHAMED KAMAL EL-DIN FOUAD
BORN AT CAIRO (EGYPT), THE 16TH OF FEBRUARY 1924

H. VEENMAN & ZONEN - WAGENINGEN - 1956

To my father
to my mother
and to my wife

Dit proefschrift verschijnt tevens in de Mededelingen van de Landbouwhogeschool te Wageningen (Nederland) **56** (10) 1–55 (1956), en als Mededeling 163 van het Laboratorium voor Phytopathologie, Wageningen.

ACKNOWLEDGEMENTS

This work was carried out under the direction of Prof. Dr. A. J. P. OORT, to whom I am deeply indebted for his guidance and critical advice.

The suggestions and the continued interest of Dr. M. H. VAN RAALTE are greatly appreciated.

I should like also to express my thanks to Dr. Ir. A. P. KOLE, Mr. F. SCHEYGROND and Mr. B. J. VAN TONGEREN for their help in preparing the photographs and the figures.

The author acknowledges the assistance of the T.N.O. Research Team for Internal Therapy of Plant Diseases in providing the G 33.

CONTENTS

PART II

GENERAL INTRODUCTION

The problem of host-parasite relationship in hosts susceptible or resistant to plant diseases, has been studied since the second half of the last century. The literature in this respect is numerous, but the writer finds it of great value before mentioning the aim of the present work, to review as briefly as possible the most important work which was carried out and the conclusions reached by a number of authors in this field.

In 1886 DE BARY (7) announced the view of "killing in advance of penetration" after studying the mode of invasion of *Sclerotinia libertiana* in infected tissues of plants as broad bean, carrot, etc. This view was confirmed by other authors with other fungi However, they did not prove that the fungal extract had a similar activity. BROWN (16) reported that with *Botrytis cinerea* the infecting germ tubes or their strongest extracts are unable to affect chemically the cuticle of the host, nor do they secrete any toxic substance which can pass through the cuticle and bring about the death of the underlying cells. He concluded that either an important principle had not been extracted, or that "killing in advance of penetration" claimed by DE BARY was unfounded at least for *Botrytis cinerea*. Cytological investigations carried out by several authors (10, 13, 26) with different fungi including *Sclerotinia libertiana* supported the adverse view, viz. that penetration preceded killing of the underlying host cells.

Penetration of the host surface is accomplished in 3 ways; directly through the epidermis, through stomata or lenticels, or through wounds. A theory of positive chemotropic attraction put forward by MIYOSHI (49, 50) according to which the germ tubes grow towards the source of some attracting substances diffusing out of the host cells, was opposed by FULTON (30) who stated that negative chemotropism is the rule. As to the application of these two diverse theories in explaining the entrance of a fungus into its host, much work has been done to prove either of these contradictory theories. On the one hand, for instance, MASSEE (47) recorded that "an immune plant is an individual of the same species as the one on which a given species of fungus is parasitic, but which, owing to the absence of the chemotropic substance in its tissues necessary to enable the germ tubes of the fungus to penetrate, remains unattacked." On the other hand BROWN (18) denied any dependence of the penetration process on either of the two different tropisms.

BROWN and HARVY (17) in their work on the entrance of parasitic fungi into the host plant, concluded that the only satisfactory theory of membrane penetration by fungi is; (a) the stimulus to penetration is one of contact, (b) the means of penetration is purely mechanical.

As to the mechanical penetration of the host surface, there is convincing evidence that the increase in thickness of the cuticle, skin, etc. of the plant body causes increased resistance to some diseases (25, 35, 48, 68).

In relation to stomatal penetration, the so-called "functional resistance" has been reported by POOL and MCKAY (55) for mature beet leaves against *Cercospora beticola*, and by HART (36) for wheat varieties against *Puccinia graminis triciti*. The second author mentions the slow opening after sunrise and the early closing of stomata of some highly resistant varieties as the actual cause for resistance. This is due to the fact that the critical period of stem-rust infection is in the early morning immediately after sunrise and while the plants are heavy with dew.

The size of stomata may act in resisting the stem-rust fungus, as found by ALLEN (2). She noted that the rust "sporeling" did not enter freely through the naturally small stomatal openings of the resistant wheat variety she examined.

BROWN (19) states that the facultative type of parasite illustrated by such a typical example as *Botrytis cinerea*, invades the tissues of the host by excreting a destructive principle which kills the cells and more or less dissolves the cell walls in advance of the hyphae. He added that early and rapid killing of the host tissue is not always so pronounced as with *Botrytis cinerea*, and in some cases

killing is slow and not at all distinct at first. There are thus intermediate types which lead up to the condition shown by obligate parasites such as *Puccinia graminis* when growing in symbiosis with its appropriate host. He also added that one cannot ignore the possibility that fungi may be able to progress through a tissue by mechanical action alone. Cell walls might be pierced by mechanical means and the mere presence of a foreign body, such as a hypha within the protoplast, might conceivably lead to death of the latter. In his opinion, where there is pronounced rotting of the tissue, chemical action is doubtless predominant, and conversely, where microscopic study shows that the fungus has traversed cell walls by means of fine penetration hyphae, one is justified in speaking of mechanical penetration.

As to the destructive principle, BROWN (15) claimed that it is the enzymic system, in particular the pectinase (the enzymic theory), while CLAYTON (22) and PIERSTORFF (54) ascribed it to an unspecified thermostable substance, HIGGINS (40) and JOHANN et al (42) to oxalic acid (the acid theory).

BROWN (19) pointed out that in obligate parasites the thallus of the fungus is entirely intracellular (as in *Synchytrium* or *Plasmodiophora*) or the haustauria are the only intracellular structures (as in powdery mildews and rust fungi). In facultative parasites the hyphae are inter- as well as intracellular.

The mechanism of resistance after successful penetration into the resistant hosts differs widely. A mechanical type of resistance is reported by several authors. For instance the mechanical hardness of tissues is stated by WILLAMAN (76) to be the cause of resistance of plum trees to *Sclerotinia cinerea*, whereas HAWKINS and HARVEY (38) found a similar relationship between the mechanical properties of the tissues and susceptibility to *Pythium debaryanum*. In the last case it was shown that resistance to infection in resistant tissues is correlated with a higher crude-fibre content which was considered to be due to more secondary thickening in the cell walls. Also the investigation of HURSH (41) illustrates a clear example. He found that some wheat varieties in which there is a great deal of sclerenchyma are less likely to be injured by stem rust, as there is a mechanical limitation to the spread of the mycelium, since the latter can only grow in the chlorenchymatous tissue of the stem, i.e. the collenchyma. To this he attributed also the fact that the seedlings of such varieties are more susceptible than the older plants.

The formation of cork layers plays an important role in the mechanical resistance. SHAW (63) declared after studying resistance of apple and Rosaceous plants to fire-blight, that once the fire-blight lesions are corked off, the cork layers commonly serve as relatively effective barriers against further invasion by the pathogen. The more resistant the variety to fire-blight, the shorter the time required for the corking-off of the blight lesion. CUNNINGHAM (24) reported in his study of the histologic changes induced in leaves by certain leaf-spotting fungi, that leaves of *Prunus domestica*, *P. cerasus*, and *P. virginiana*, when attacked by species of *Coccomyces*, form a definite cicatrice about the edge of the lesion, thus isolating the diseased portion from the healthy. The same reaction occurs when leaves of *Pyrus communis* are attacked by *Mycosphaerella sentina*, and when leaves of *Beta vulgaris* are attacked by *Cercospora beticola*. Artificial wounding of leaves of those plants, results in the formation of a cicatrice similar to that found in diseased leaves. In tobacco root rot caused by *Thielavia basicola* CONANT (23) found that sections through lesions from plants grown in infested soil show a distinct correlation between resistance and cork

formation in tissues underlying the lesions. Very susceptible varieties show no cork formation whereas resistant varieties show strong cork formation. BONDE et al. (12) claimed that the resistance of tubers, of potato varieties which are resistant to *Phytophthora infestans*, is due to the presence of a thick protective periderm. BROWN (20) in a very recent publication stated that cork is rather the sequence than the cause of resistance. He added that a cork barrier, in spite of its prominence, is of no greater significance than "a monument on a battlefield".

Gum formation constitutes another mode of mechanical resistance. BROOKS (14) studied the silver leaf disease caused by *Stereum purpureum*, and found that in conditions favourable for gum formation, the wood of the host will in advance of the fungus produce so much gum in a relatively short time, that the fungus becomes completely enclosed by an impassable gum barrier. Within this barrier the fungus may continue to live for a considerable time, but eventually it dies. ABDEL-SALAM (1) working on *Botrytis* disease of lettuce, examined unstained hand sections and observed a wide band of cells free from fungal elements, which were of a brown discolouration with thick walls. The appearance suggested that there is a deposition of gum-like substance on the cell walls. To this band of cells the investigator attributed resistance of the host tissue.

GÄUMANN (31) in his publication on types of defensive reactions in plants, considered cork and gum formation as antitoxic and not as anti-infectional reactions; the reaction is not incited by the parasite itself, but by the necrosis caused by it. The invading hyphae may or may not pass these cork or gum defensive demarcations.

Hypersensitivity, i.e. necrosis of small spots in host tissues around the seat of infection so that the required food is not available, is well known in obligate parasites. ALLEN (3) stated that in "Kanred", a wheat variety resistant to stem rust, a small haustorium is formed within the host cell, but soon either by its presence or, as is more likely, by the secretion of some substance, a chemical reaction started within the host cell causing its collapse and death. A similar case was also found by ALLEN (4) when she studied the reaction of "Mindum" a wheat variety to *Puccinia graminis tritici* form III. WELLENSIEK (74) in his study on the nature of resistance in *Zea mays* to *Puccinia sorghi*, reports that in the resistant host the development of the fungus is much slower and does not progress as far as in the susceptible host. Only a few spores are formed and a general necrosis of both host cells and fungus follows immediately. He assumes that the amount in which a nutrient substance is present, determines the degree of susceptibility. The antagonistic reaction between host and parasite, recorded by SMITH (65) in clover plants resistant to *Erysiphe polygoni*, soon results in the collapse of the invaded epidermal cells.

A very recent work carried out by PRISTOU and GALLEGLY (56) shows that hypersensitivity may also occur in facultative parasites. They investigated leaf penetration by *Phytophthora infestans* in susceptible and resistant varieties. After successful penetration into the resistant leaf, only pin-point necrotic spots developed on the leaf surface. In sections of such a leaf, the mycelium was never observed beyond the epidermal cells originally penetrated. Disintegration of the primary mycelium and death of cells surrounding the point of penetration occurred between 48 and 72 hours after inoculation. On the contrary typical spreading-type lesions developed on a susceptible leaf after the finger-like secondary mycelium had spread freely throughout the host tissue.

Internal chemical resistance of the host tissues has been revealed by several

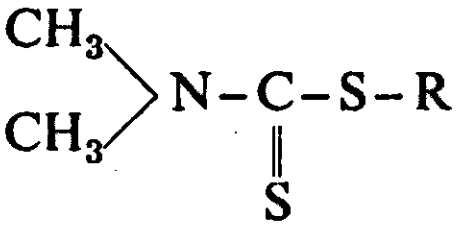
authors (37, 46, 57, 58, 60) on the basis of the presence in the cell sap of a substance toxic or inhibitory to the invading pathogen.

The relation of resistance to host metabolism, was very recently revealed by GOTHOSKAR *et al.* (32). They postulated that resistance of the tomato plant to *Fusarium lycopersici* is closely related to the metabolism of the plant, and is probably due to a very labile substance continuously formed at the expense of energy obtained from respiratory processes.

The appearance of resistance of a host to a parasite as it ages, whereas the seedlings of that host are susceptible, is recorded by different investigators (27, 33, 34, 44, 64), hence the so-called "mature plant resistance" factor was revealed.

The aim of the present study was to investigate the host-parasite relationship in susceptible and in resistant cucumber tissues (*Cucumis sativus* L.) infected by *Cladosporium cucumerinum*, the cause of cucumber scab.

Induced resistance could also be studied, because a substance was available, which is translocated in the plant and which protects the plant against infection to a considerable degree. This substance was discovered in the course of experiments carried out by VAN RAALTE *et al.* (72) on systemic fungicides. The fungicide Tetramethylthiuram monosulphide TMTM, was shown by VAN RAALTE (71) to be readily translocated through plant tissues, but unfortunately failed to show any chemotherapeutic activity. This together with the fact that the activity of TMTM is considered by KLÖPPING and VAN DER KERK (45) to be due to the dimethyldithiocarbamate ion, led VAN RAALTE *et al.* (72) to attempt to obtain the latter as transportable compounds that show chemotherapeutic activity. They tested a number of synthesized compounds with the general formula:



in which R was a hydroxyethyl-, carboxymethyl-, benzyl- or acetonyl- radicle etc.

One of these compounds was S- carboxymethyl-N, N-dimethyldithiocarbamate, which was indicated by the authors with its code number G 33.

The authors found that if this compound is applied to the roots of cucumber plants, the tops (the above ground parts) become more or less resistant to *Cladosporium cucumerinum*. The compound was proved to have little fungicidal activity in vitro. The investigators found it more suitable to describe it as a systemic or internal protectant.

Since the mechanism by which the effect of G 33 is brought about, is still obscure, this problem was involved in the present investigation.

Genetic resistance of two scab-resistant varieties was also investigated, and compared with the G 33 induced resistance.

The previous work on susceptibility and resistance of cucumber tissues to *Cladosporium cucumerinum*, will be mentioned in the following pages when describing or discussing results.

PART I

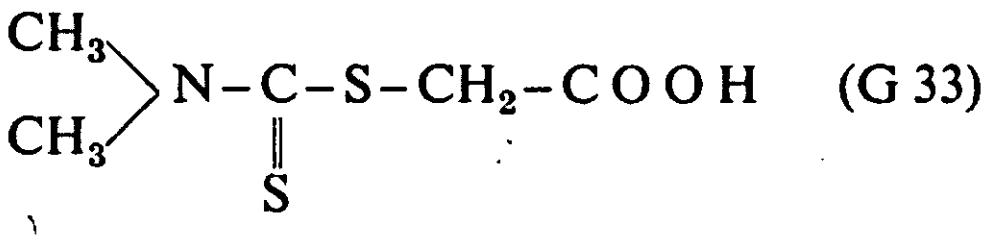
MICROSCOPICAL STUDIES ON HOST-PARASITE RELATIONSHIP IN CUCUMBER TISSUES, SUSCEPTIBLE, G 33-RESISTANT, AND GENETICALLY RESISTANT TO CUCUMBER SCAB

CHAPTER I

THE DIFFERENCE IN REACTION TO THE CAUSAL ORGANISM BETWEEN SUSCEPTIBLE SEEDLINGS, UNTREATED AND TREATED WITH G 33

A. INTRODUCTION

The aim of this chapter was to investigate microscopically the effect of S-carboxymethyl-N, N-dimethyldithiocarbamate,



(G 33)

on cucumber seedlings inoculated with *Cladosporium cucumerinum*, the cause of cucumber scab.

If a solution of this compound, which will be indicated in the following pages by its code name, G 33, is applied to the root system of a cucumber plant, the top of the plant becomes more or less resistant to the disease.

The behaviour of the fungus in treated and non-treated cotyledon tissues was studied and the results are discussed.

B. MATERIALS AND METHODS

Seeds of the susceptible variety "Lange gele tros" were sown in sand. The pots were placed in the greenhouse. Seven to ten days after sowing the seedlings were used for the experiments. By that time they consisted of a root system, a hypocotyl, two cotyledons and a growing point.

The seedlings were then carefully lifted, and their roots were rinsed to remove the adhering sand grains. Groups of 5 plantlets were placed in 50 ml. wide-mouth bottles. These bottles were filled to a height of about 2 cm. with distilled water, or with a solution of 100 p.p.m. of G 33. Only the roots and the lower part of the hypocotyl were in the fluid. After 48 hours the seedlings were taken out of the bottles, rinsed under the tap and placed in fresh distilled water. They were then inoculated, by spraying with an aqueous spore suspension from a De Vilbiss atomizer. A second method to inoculate the seedlings, was to make two lengthwise scratches in the cotyledons, by wounding the upper epidermis at both sides of the midrib with a blunt needle. The scratches were then filled with an aqueous spore suspension from a glass capillary.

The temperature in the greenhouse where the seedlings were grown varied according to the weather-conditions outside. After inoculation, however, the plantlets were placed in glass boxes in a greenhouse, the temperature of which remained constant at 20 °C. A high humidity was maintained by placing bricks

soaked with water in the glass boxes. Moreover, for the first two or three days after inoculation the seedlings were covered with a sheet of thin polyethylene plastic.

From the second day after inoculation small pieces of tissue were daily taken from the cotyledons of treated and of non-treated seedlings. These were fixed by immersion in formalin-propiono-alcohol (90 ml. of 70% ethylalcohol, 5 ml. of propionic acid, 5 ml. of 40% formaldehyde solution). The air was removed from the intercellular spaces of the tissue by evacuation with a waterpump.

Free hand sections were made, which were stained in cotton blue-lactophenol and mounted in concentrated glycerine.

The examination of slides of hand sections was made with a bright field microscope[1]. Some observations on the cell contents and the cell structure in infected tissue were made using a phase contrast microscope[2].

The figures of the present and the following chapters are Camera lucida sketches.

C. RESULTS

1. *Relation of the pathogen to cotyledons of the non-treated seedlings*

In the non-treated seedlings, the spores germinated on the surface of the cotyledon within 48 hours, developing germ tubes which ended in well-defined appressoria (fig. 1). Soon after the appressoria were formed direct penetration of the cuticle took place. In most cases, after passing the cuticle, the hypha grew in a direction perpendicular to the surface between two epidermal cells. Occasionally, however, a hypha grew parallel to the surface in the outer wall of the epidermal cells, just below the cuticle, for a considerable length before it started growing inwards (fig. 2). Much less frequently, penetration took place through a stoma (fig. 3).

Over the frequency of penetration through the stomata, the data in literature do not agree. Stomatal penetration was not found by PIERSON and WALKER (53), whereas BOND (11) reports, on the contrary, that penetration by way of the stomata was frequently observed.

The mode of penetration via the cuticle by *Cladosporium cucumerinum* was not definitely ascertained before. A very close examination, made by the author of many preparations, showed that there was no microscopic evidence of the cuticle being softened or swollen or in any way altered chemically. It is, therefore, highly probable that the infection hypha from the adhering appressorium might pierce the cuticle by means of mechanical pressure.

After passing the epidermis the hyphae usually grew without much branching between the palisade cells in the direction of the spongy parenchyma. In the large intercellular spaces of this tissue the mycelium developed and spread more rapidly than in the narrow intercellulars of the palisade parenchyma. The mycelium remained intercellular as long as the host cells appeared healthy. Intracellular hyphae did not develop before the collapse and necrosis of the cells of the host tissue. One of the most conspicuous features of the intercellular mycelium in the invaded tissues was that the majority of the hyphae were in close contact with the cell walls (fig. 4), often following every curvature of the surface of the cells. If a hypha that crossed an intercellular space met a cell wall

[1] Zeiss-Winkel Standard-Microscope, oc. 6× & 12×. obj. 10× n.a. 0.25 & 40× n.a. 0.65.

[2] Phase Contrast Microscope "Nedoptifa", Zeist, The Netherlands, oc. 10×, obj. 40× n.a. 0.65.

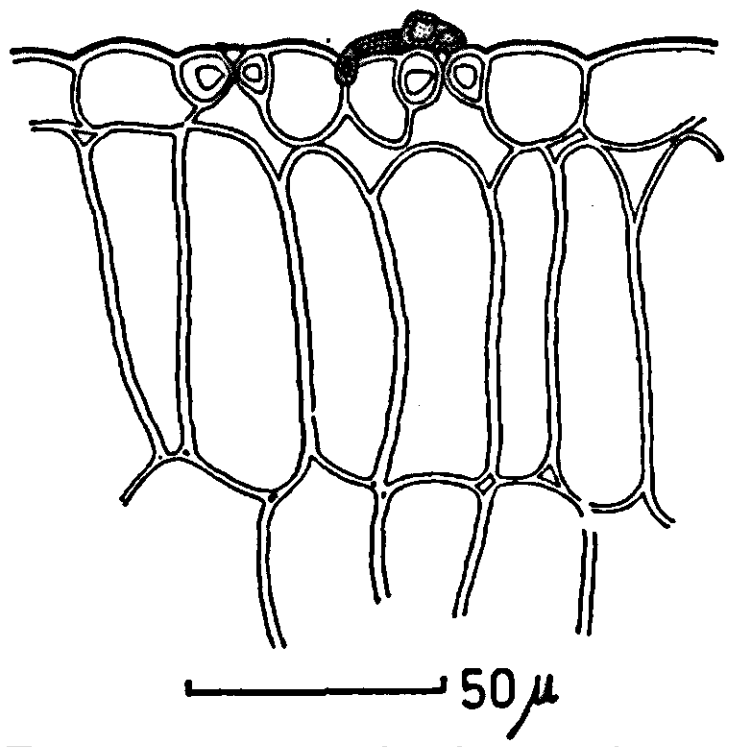


FIG. 1. Spore germination and formation of a well-developed appressorium on the epidermis of a susceptible cotyledon.

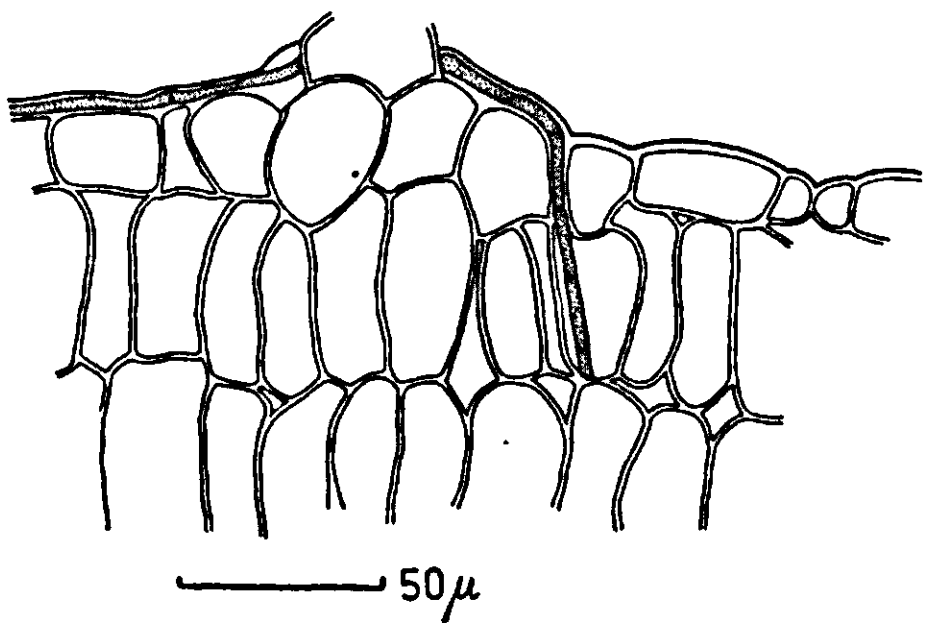


FIG. 2. Direct penetration into a susceptible cotyledon. Note the subcuticular growth of the hypha before its penetration between two epidermal cells.

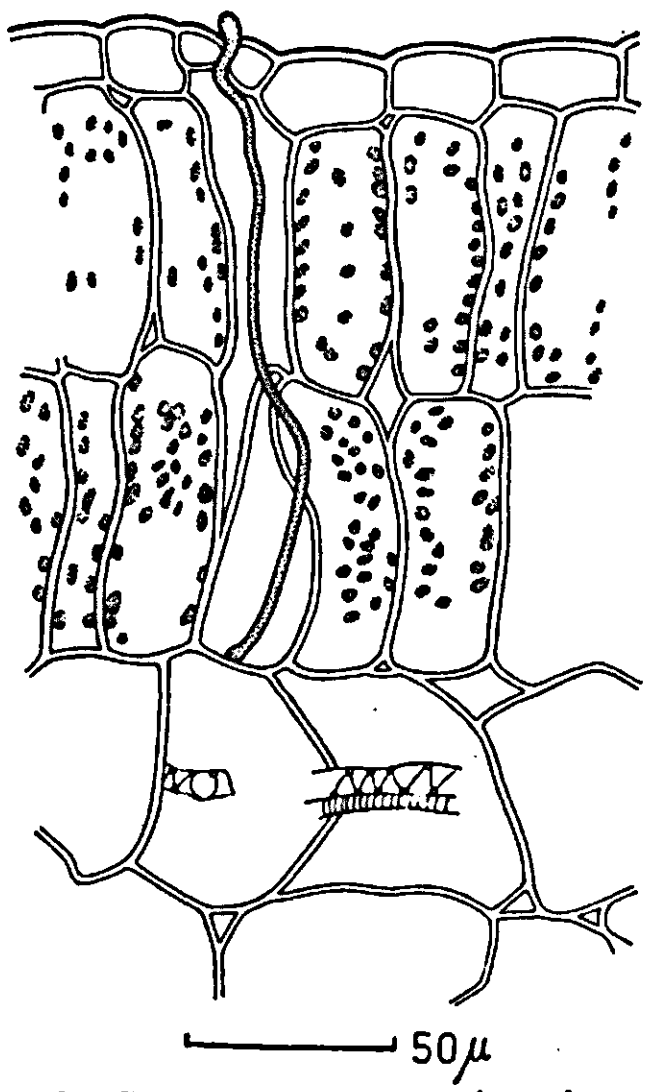


FIG. 3. Stomatal penetration into a susceptible cotyledon.

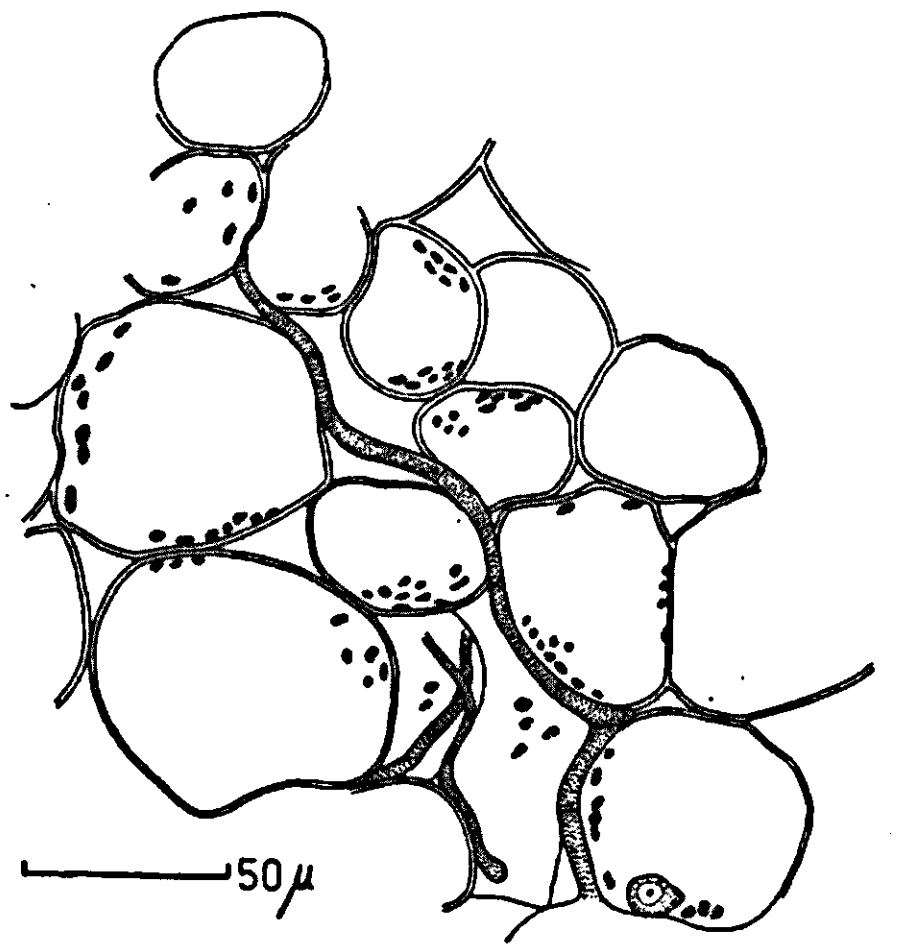


FIG. 4. Infected spongy tissue of a susceptible cotyledon 5 days after inoculation with the intruding hyphae growing intercellularly, mostly in close contact with the cell walls. The host cells are not visibly affected during this intercellular habit of the fungus.

more or less at a right angle its tip was usually markedly swollen at the place of contact (fig. 5). No structural changes in the cell wall of the host cells were found during the stage of intercellular growth of the fungus.

In advanced stages of infection many cells of the palisade parenchyma show peculiar structures. These appear as highly refractive irregular rings or ellipses in the lumen of the cells (Plate I; Photo 1). Careful study

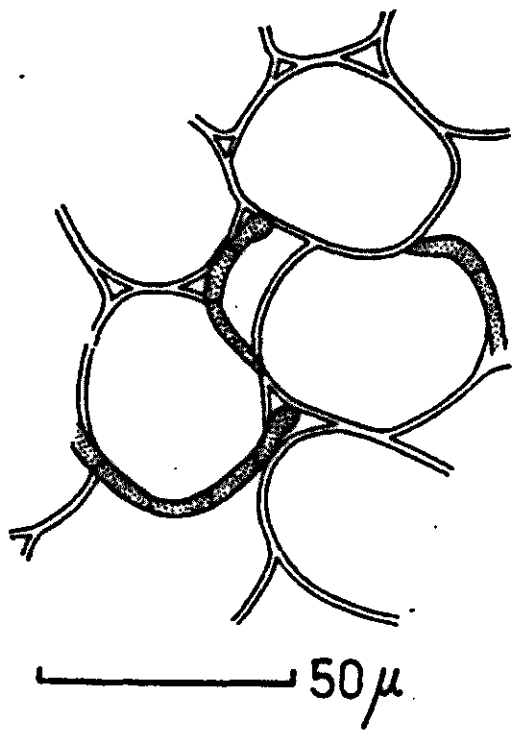


FIG. 5. Swelling of the tips of the intercellular hyphae as they meet the cell walls more or less at right angles.

of thin hand sections revealed that these structures were not parts of the cell contents, but that they occurred on the walls on the outside of the cells. In one case, in a section which had been treated with zinc chloride iodine, a flap of blue stained cell wall material was found attached to the wall of an intact cell, exactly at the place where the latter showed a ring.

From these observations it became clear that these rings or ellipses, mark the circumference of the contact surface between the walls of two adjacent cells. In healthy cells these structures are not visible because the walls of the adjacent cells are nearly completely united (fig. 6). Only at the edge of the cells the walls separate to form the narrow prismatic intercellular spaces, which are common to nearly all parenchyma cells.

Apparently in the infected tissue, the pectin of the middle lamella is dissolved, starting from the intercellulars at the edge of the cells. Probably this is due to a propectinase excereted by the fungus. The cells shrink a little thus the intercellular spaces increase considerably in size (fig. 7). Only in a small zone the cell walls of the adjacent cells remain united by the middle lamella, and it is the circumference of this zone which forms the strongly refractive ring or ellipse.

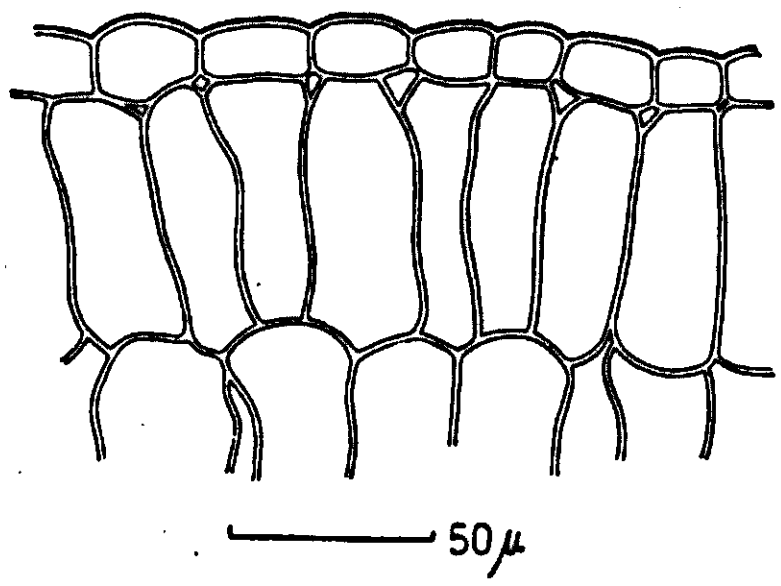


FIG. 6. A palisade tissue of a healthy susceptible cotyledon, free from the peculiar structures present in the cell lumina of diseased palisade tissue (photo 1, fig. 7).

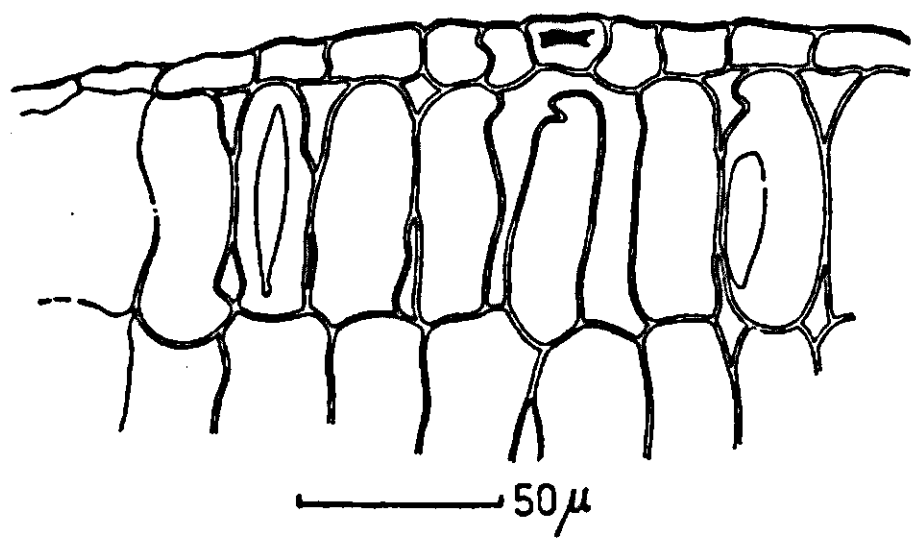


FIG. 7. Increase in size of the intercellular spaces between palisade cells of an infected susceptible cotyledon, due to partial solution of the middle lamella, and slight shrinkage of the cells. Note the peculiar structures in the lumina of several cells.

Many cells of the spongy parenchyma show ring-like structures in their lumina, in both healthy and diseased tissue. These rings represent the places of wall contact of the adjacent spongy cells which possess naturally big intercellulars. Here, it is possible that the assumed pectolytic enzyme excreted by the fungus also dissolves parts of the middle lamella, and this might cause a slight not easily detectible increase in the size of the already big intercellulars.

The rate at which the mycelium spreads within the diseased tissue, differed much in the successive experiments. The hyphae from spores on the surface of the cotyledon reached the opposite epidermis after a period of 3–6 days after inoculation. The differences in the rate at which the disease spread through the cotyledon may possibly be ascribed to the different environmental conditions which prevailed in the greenhouse where the seedlings were raised.

In the final stage of the disease there was increased ramification of the hyphae. The cells of the host tissue collapsed and necrosis occurred. It was only then that the intracellular hyphae were observed. The conidiophores arose in a massive quantity from the collapsed upper surface of the diseased tissue. They emerged

in a smaller amount from the lower surface, where the epidermis was often much less damaged. In the last case, the conidiophores grew either through the stomata to the exterior, or they penetrated the cuticle (fig. 8).

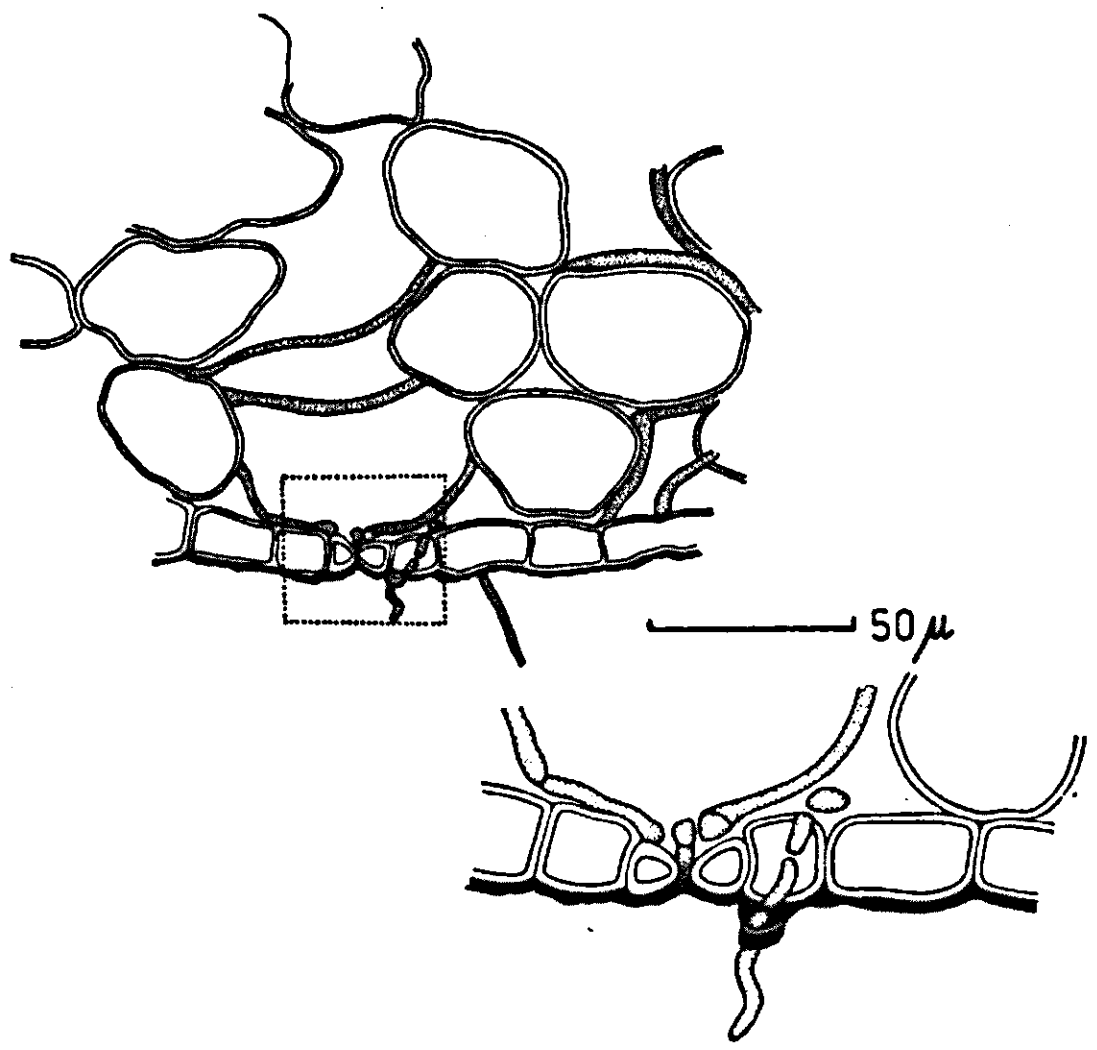


FIG. 8. Within the dotted square, the beginning of the emergence of a conidiophore from an infected susceptible cotyledon, by penetration through the cuticle of the lower epidermis, which remains nearly undamaged at the very last stage of infection. At the lower right corner the same stage is about two times magnified.

"Gum" was occasionally present, as a yellow non-granulated substance, in the intercellular spaces between intact cells, and, sometimes in the interior of the cells, in very small amounts. BEHR (8) states that the gum which is secreted by diseased cucumber tissue is formed by the breakdown of the host cells. This could not be confirmed in the present investigation. The large gum-filled pockets which are described by PIERSON and WALKER (53) were not seen.

In the non-treated and wounded cotyledons, which had been inoculated by placing a spore suspension in the scratches, the course of the development of the disease in the tissues was the same as described above.

2. *The pathogen in cotyledons of the treated seedlings*

In the cotyledons of seedlings which previously to inoculation had been treated with G 33, there was no effect of the treatment upon the germination of the spores on the intact epidermis, and upon the formation of appressoria. The latter failed, however, to develop any hyphae, and therefore, no penetration and no subsequent infection of the host tissue took place. Only one case was observed in which an appressorium had succeeded to develop a hypha. This hypha ended in the aperture of a stoma and, apparently, had ceased its growth here (fig. 9).

It was first thought that the cuticle had been only affected by the treatment, and therefore, might act as an efficient barrier to the penetration process. It was also assumed that once the cuticle is removed, the influence of G 33 will disappear and the underlying tissues will be naturally infected. The whole

hypothesis was disproved by studying the course of infection in cotyledons where inoculation took place by the deposition of spores in a wound. Here the cuticle had been previously removed by scratching. The spores germinated in the scratch as well as on the intact surface, and formed normal appressoria. The outstanding difference with the development of the fungus on the intact surface was that in the scratches, the appressoria developed hyphae, which grew in the dead cells bordering the wound till the intact tissue was reached. Here the hyphae failed to advance in the intercellular spaces further than 1–2 layers of cells beyond the damaged area (fig. 10).

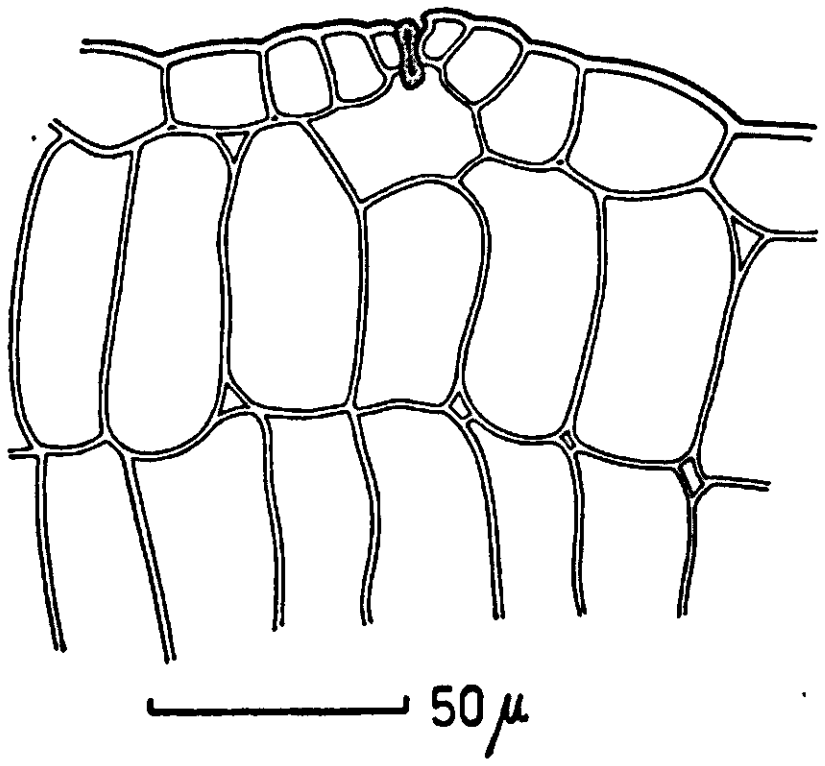


FIG. 9. A rare case in which penetration into a cotyledon of a susceptible seedling treated with G 33 took place. An appressorium had developed a hypha that penetrated a stoma, and soon ended its growth in the stomatal aperture.

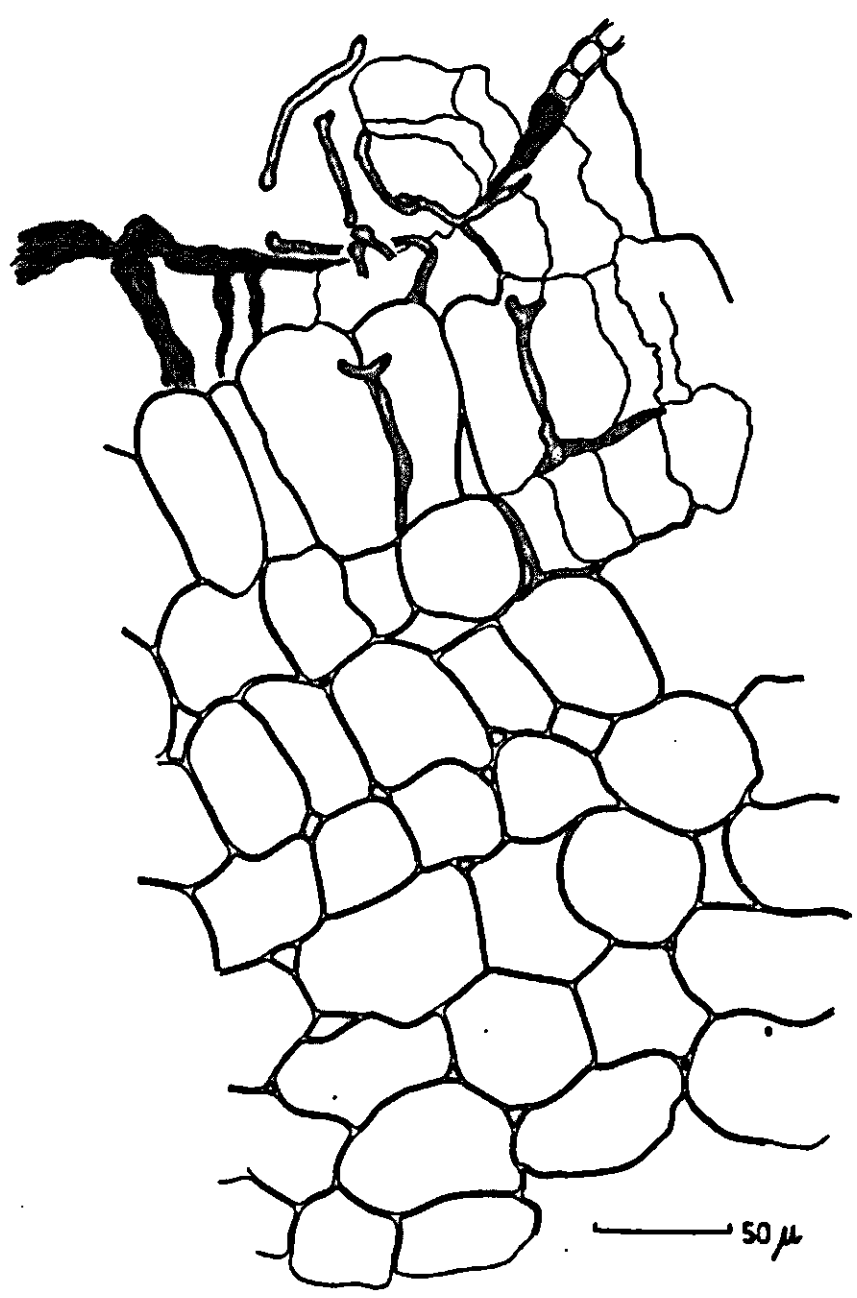


FIG. 10. Part of section from a G 33-treated susceptible cotyledon, the surface of which was scratched and the wound inoculated. After normal spore germination, hyphae from appressoria penetrated through the dead cells of the wound and then intercellularly between the adja cent healthy looking cells, where they failed to proceed further than 1–2 cell layers.

It can be concluded then that: 1. the influence of G 33 is not only restricted to the cuticle, 2. the interaction between G 33 and the host cells is only effective in impeding the advance of the hyphae if the host cells are at least microscopically intact.

The un-restricted effect of G 33 was discovered by VAN RAALTE *et al.* (72) through their macroscopical observations on the location of the increased resistance induced by treatment with G 33.

3. *Microchemical reactions*

Some microchemical tests were carried out to investigate: *a.* the effect of G 33 on the chemical composition of the host cell walls, *b.* the nature of the secreted substance in diseased cotyledon tissue.

To detect pectic substances, the cells were stained with ruthenium red in the way indicated by JOHANSEN (43). Zinc chloride iodine was used as an indicator for cellulose.

Pectic substances. In sections of the cotyledons from seedlings which had not been inoculated with the fungus, the cell walls of the parenchyma cells stained evenly with ruthenium red. Heating of the sections for 20 minutes in 2 % aqueous hydrochloric acid caused a chemical change of the pectin by which the property of the cell walls to take the red stain disappeared. The cell walls of these sections remained unstained or obtained at most a faint pink colour. In these respects no differences were found between the seedlings treated with G 33, and the non-treated controls. This indicates that the treatment with G 33 has no direct influence on the pectin of the cell walls.

Inoculation of the seedlings with *Cladosporium cucumerinum*, on the contrary, had a remarkable effect on the pectin of the cell walls. If infected sections were placed in ruthenium red without pretreatment, the staining of the parenchyma cells was not different from that of the uninoculated seedlings described above, viz. the cell walls in all parts of the section stained red with equal intensity. If, however, the sections were pretreated with hot 2 % aqueous hydrochloric acid the cell walls lost the capacity to take the red colour only in some parts of the section whereas in other parts the cell walls were stained after immersion in the ruthenium red solution. Such sections showed alternate red and unstained patches. The red patches being manifested by parts from sections where the hyphae were present, while the unstained patches were not yet reached by the fungus.

Sections pre-treated with acid from cotyledons in which the fungus had been introduced in a scratch, stained red in a zone around that scratch even when, owing to treatment with G 33, the hyphae had only been able to penetrate through a very few layers of the intact cells.

These observations show that the presence of the mycelium in the cotyledon tissues changes the chemical or the physical properties of the pectin of the cell walls, in such a way that it becomes insoluble in acid.

Cellulose. With zinc chloride iodine no differences were found between the reactions of the cell walls in cotyledons from treated and from untreated seedlings. The presence of the mycelium in the tissue caused no difference in the cellulose reaction even at places from the cell wall where it was in close contact with a hypha.

"Gum". BEHR (8) showed that the gum formed in the diseased tissues was a true wound gum. VAN DE MUYZENBERG (52), and PIERSON and WALKER (53) also mentioned the presence of gum in the infected tissues. The "gum" which was found in the present investigation occurred in small amounts within the cells or in the intercellular spaces. It did not stain either with ruthenium red or with zinc chloride iodine, showing that it had neither a pectic nor a cellulose nature.

D. DISCUSSION

The present work has shown that in non-treated cotyledon tissues the fungus spreads rapidly through the tissues. In tissues where the cells had not yet col-

lapsed the mycelium remained intercellular. This was found also by BOND (11), whereas PIERSON and WALKER (53) state that in diseased leaf tissues from 96 hr. after inoculation onwards, intracellular hyphae frequently occur. Probably in the present investigation, the hyphae obtained their nutrients by diffusion through the cell walls with which they were in close contact.

The presence of the highly refractive rings or ellipses in the lumen of the infected palisade cells, which may be ascribed to a partial solution of the middle lamella by a pectolytic enzyme secreted by the fungus, has not been reported before in literature.

In sections from infected cotyledons, the unchanged capacity of the cell walls to stain with ruthenium red after treatment with hot 2% HCl. only in those parts occupied by hyphae, is a striking result. This may possibly be due to a secretion by the pathogen of a substance which changes the chemical or the physical state of the pectin of the cell walls and the middle lamellae, rendering them insoluble in acid.

It is evident from this study that the decreased growth of the fungus in the tissue of plants treated with G 33 as compared to that in non-treated plants is not due to a plasmatic interaction. The hyphae are not in contact with the host protoplasm until the very last stage of the disease. The only positive statement which can as yet be made on the mechanism of action of G 33 is, that the treatment of plants with this substance causes an inhibiting principle to be present in the cuticle, the cell walls and the intercellular spaces. Only a small contact surface between the hypha and the cell wall is required for inhibition of hyphal growth as shown by fig. 10. Here the hypha touches only the two guard cells of the stoma. Unless one assumes an inhibiting gas to be present in the intercellulars, this hypha has been arrested in its growth owing to a substance diffusing out of these cells.

CHAPTER II

THE BEHAVIOUR OF THE PATHOGEN IN COTYLEDONS OF CUCUMBER SEEDLINGS OF THE TWO SCAB-RESISTANT VARIETIES "MABRO" AND "PROSO" [1])

A. INTRODUCTION

To meet the requirements of cucumber growers, obtaining scab-resistant varieties was, and still is the only efficient way of controlling the disease. Most of the work in this field was on the practical side of the problem rather than with the histological basis of genetic resistance.

In Holland, VAN DE MUYZENBERG (52) reported that most varieties investigated were susceptible. A few were less susceptible, these were slow growing varieties. Anatomical investigation of the leaves of various varieties revealed only slight differences in thickness of the cuticle and in the size of the epidermal cells. In Germany, SCHULTZ and RÖDER (62) tested 104 varieties for field resistance during 3 subsequent years. The two varieties "Delikatesz" and "Dickfleischige lange grünbleibende" were nearly resistant under field conditions. In the United States, BAILEY and BURGESS (5) started an inbreeding pro-

[1]) The seeds of these varieties were kindly supplied by Ir. G. W. van der Helm, Rijkstuinbouwconsulent te Amsterdam.

gram with plans for subsequent testing of the resistance and selection of inbred lines. They selected 4 to 12 successful self-pollinations of 22 varieties of cucumbers, and this produced a total of 125 seed lots. Of these lots 117 exhibited no resistance, while the remaining eight gave an indication of resistance of different degrees. This was demonstrated either in the seedling stage in the greenhouse, or in the fruit bearing plants in the field. According to PIERSON and WALKER (53) BAILEY (6) developed the resistant variety "Maine No. 2", by using the variety "Longfellow" as a source of resistance. WALKER, PIERSON and WILES (73) initiated a breeding program in which "Maine No. 2" was used as the resistant parent in crosses with "National Pickling" and "Chicago Pickling", the standard varieties generally in use. At the end of this program they succeeded in raising two resistant varieties, "Wisconsin SR 6" to replace "National Pickling", and "Wisconsin SR 10" to replace "Chicago Pickling".

The only considerable microscopical study of the nature of genetic resistance was carried out by PIERSON and WALKER (53) with the resistant variety "Maine No. 2". They reported that the outstanding feature of the resistant tissues is a series of host-parasite interactions, which produces cell wall thickening and cell necrosis. This feature, which has not been reported previously in cucumber, appears to be the mechanism which confines the disease to a relatively small number of host cells and prevents the formation of large lesions, which are characterestic of the disease in susceptible tissues. They added, that there is no complete collapse of cells in the resistant host; it appears that the thickening of the cell walls prevents the collapse of affected cells.

The aim of the present chapter was to investigate microscopically the host-parasite relationship in two scab-resistant varieties "Mabro" and "Proso". According to VAN DER HELM (39), "Mabro" and "Proso" are obtained from crosses between an American scab-resistant variety "Highmoor", and two susceptible Dutch varieties, "Lentse gele" and "Piers" respectively. (The varieties "Mabro" and "Proso" have not yet been released).

B. MATERIALS AND METHODS

Seedlings of the two scab-resistant varieties in the same stage of growth as that of the susceptible seedlings of the variety "Lange gele tros", were used and both methods of inoculating the cotyledons described in chapter I were applied.

The material was collected daily and was sectioned by hand, and stained in the same way as described above.

Reactions on cellulose and pectic substances were carried out as indicated in the previous chapter.

C. RESULTS

1. *Reaction of the genetically resistant cotyledons to the pathogen*

In both resistant varieties, "Mabro" and "Proso", the spores germinated and formed well developed appressoria, whether the spores had been sprayed on the intact surface, or whether the surface had been scratched and the inoculum introduced in the resulting wounds only.

After formation of the appressoria, however, penetration rarely occurred. There was an almost general failure of the well-developed appressoria to form any hyphae as long as the surface of the cotyledon was intact. Only in one case,

in a preparation from "Mabro", a hypha was observed in the host tissue, not far from the inoculated surface. Its development had ceased soon after passing the first palisade layer (fig. 11). The section did not contain the place of penetration through the epidermis, so that it was not possible to decide whether this hypha had entered the cotyledon tissue through a stoma or by a direct piercing of the cuticle. There was no collapse or necrosis of cells in the vicinity of this hypha, except for an incomplete necrosis of the two guard cells of a nearby stoma.

In two sections from "Proso", evident stomatal penetration was found. In one of these the growth of the hypha had not proceeded further than the aperture of the stoma, whereas in the other case the developing hypha had passed the aperture, its tip being in the air cavity underneath, where it had evidently failed to grow further (fig. 12). Here also neither necrosis nor collapse of the surrounding host cells had occurred.

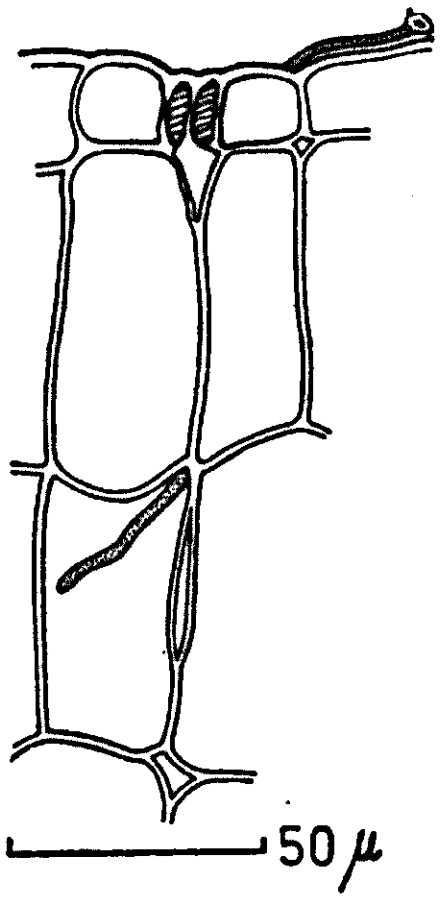


FIG. 11. A preparation from a cotyledon of "Mabro" fixed 7 days after inoculation showing a successful penetration into the host, which rarely happened. The penetrating hypha had ceased growth soon after passing the first palisade layer. All the cells in the vicinity of the hyphae except two guard cells manifested neither collapse nor necrosis.

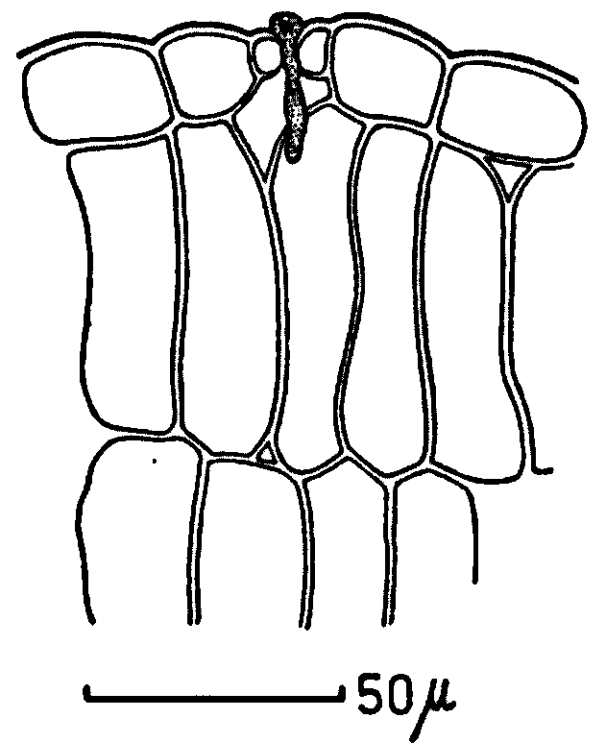


FIG. 12. A preparation from a cotyledon of "Proso" fixed 6 days after inoculation illustrating successful penetration rarely observed into the host. The developing hypha had passed the stomatal aperture, its tip being in the air cavity underneath, where it was checked to grow further. No cell collapse or necrosis did occur.

In cotyledons of "Mabro" and "Proso" the surface of which had been damaged by scratching, the fungus evidently penetrated between the dead cells bordering the wounds. Resistance, therefore, seems to be confined to the healthy cells of the host tissue.

Although the advance of the hyphae was eventually checked in both varieties there was a marked difference in the way in which this was established. In "Mabro" the hyphae penetrated through the intercellular spaces between the intact living cells until they attained, in extreme cases, the fifth or the sixth layer beyond the dead cells that bordered the wound (fig. 13). The amount of mycelium between the host cells was rather small, and no changes in these cells or in their walls were visible even in cotyledons which had been fixed 6 or 7 days after inoculation, after which time severe infection and general cell necrosis took place in susceptible tissue.

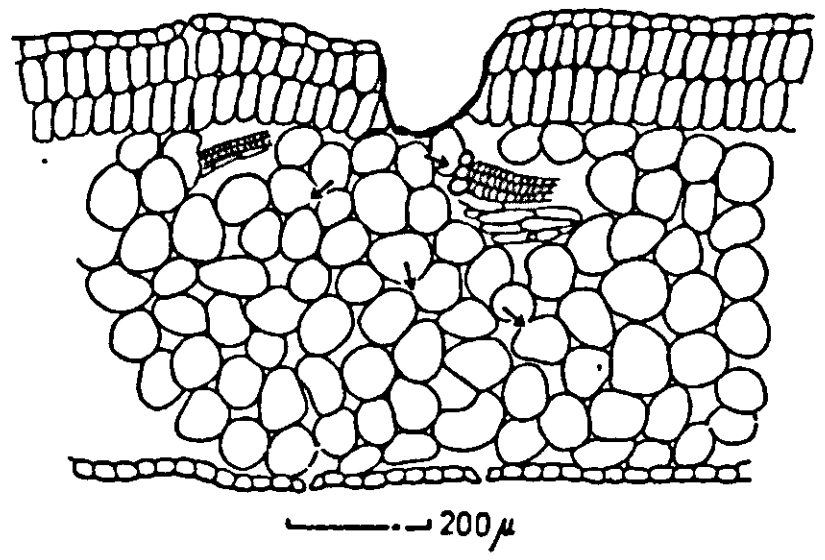


FIG. 13. A diagrammatic sketch showing infection through a wound of a "Mabro" cotyledon. The arrows point to the places within the healthy tissue reached by the intercellular hyphae originally developed from well formed appressoria on the wound surface.

The way in which in "Mabro" the fungus penetrated into the healthy tissue, from a wound, strongly resembles that which was observed in cotyledons of the susceptible variety, which had previously been treated with G 33.

In scratched and non-inoculated cotyledons of "Mabro" no wound reaction was detectable.

In non-inoculated cotyledons of "Proso", on the other hand, once a mechanical damage was caused to the tissue, e.g. by scratching as in the present investigation, a wound reaction was revealed by the secretion of a granulated yellow substance that filled mostly a few intercellular spaces between healthy cells in the neighbourhood of the damaged area (fig. 14). In addition the walls of some of these cells became yellowish in colour and no longer stained with zinc chloride iodine. The yellow substance stained with ruthenium red, and reacted positively with phloroglucin-hydrochloric acid for the indication of lignin.

In "Proso", 3–5 days after inoculation, the presence of the mycelium seemed to stimulate the secretion of the yellow substance which now filled the interior of the healthy cells as well as their intercellulars, in an almost uninterrupted zone around the wound (fig. 15). The contents of some of the cells in this zone were substituted by a yellow granular substance, whereas the cell walls were yellow in colour and slightly swollen. Within this zone, hyphae were rarely found and beyond it they were entirely absent. Evidently this zone seemed to act as a

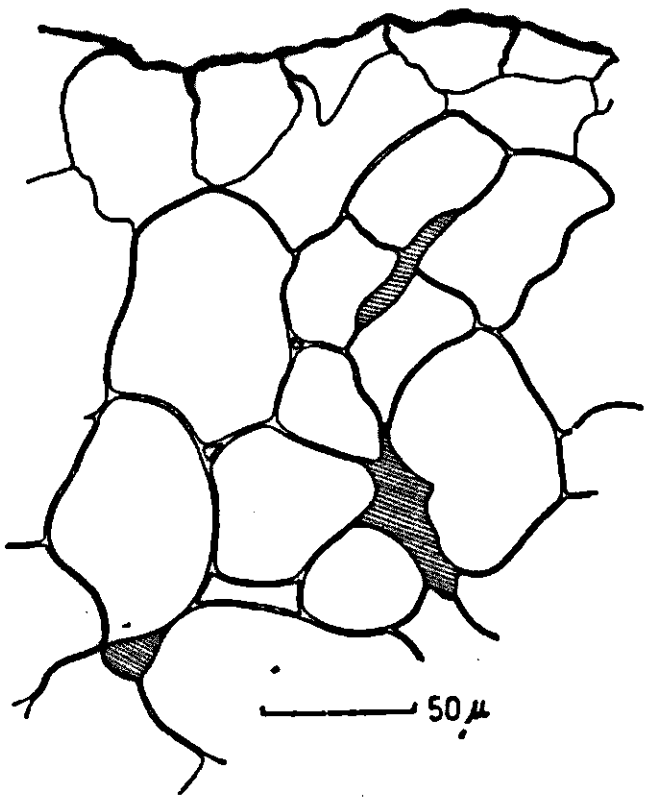


FIG. 14. A part of section from a cotyledon of "Proso", just near a wounded surface. A wound reaction is revealed by the secretion of a granular yellow substance that fills some of the intercellular spaces (the cross hatched) between healthy cells.

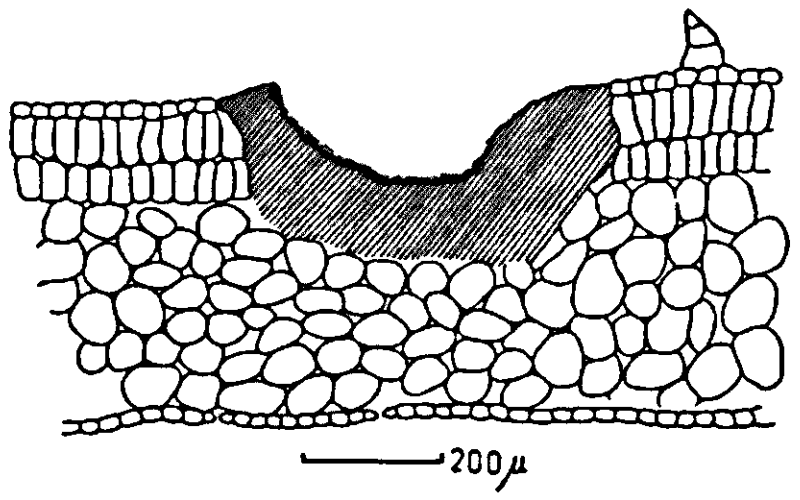


FIG. 15. A diagrammatic sketch showing infection through a wound of a "Proso" cotyledon. A zone (the cross hatched) of a secreted yellow granulated substance possibly gum, that fills the interior as well as the intercellulars of the healthy cells around the wound, seems to act as a barrier to fungal advance, after normal spore germination.

barrier against the further spreading of the pathogen. It is probable, that the formation of the yellow granular substance plays an important role in this respect.

This rather different behaviour of "Mabro" and "Proso", in checking the advance of the fungus far beyond the non-living cells bordering the wounds resulting from scratching the cotyledons, rendered it worthwile to investigate the case in scratched cotyledons of the American variety "Highmoor", the source of resistance for both "Mabro" and "Proso". Here no barrier by the secretion of any substance within or between the living cells was established, while such a barrier was, as stated above, a specific feature in scratched cotyledons of "Proso". The case was more like that in the cotyledons of "Mabro". The only difference was that in the latter the spreading of the mycelium between the living cells was somewhat deeper than that in cotyledons of "Highmoor". Here the hyphae, however, did not grow further beyond the second, sometimes the third layer of living cells around the wound.

It may be concluded, then, that the variety "Highmoor", which is the source of resistance for both "Mabro" and "Proso" has an intermediate position between the two, as far as the extent of hyphal growth of the parasite beyond the dead cells of the wounds in scratched cotyledons, is concerned.

2. *Microchemical reactions of "Mabro" and "Proso"*

Cell walls of uninoculated cotyledons and of cotyledons fixed after inoculation of both resistant varieties, were tested for the presence of pectic substances and cellulose.

Pectic substances. Heating of the intact sections in 2% aqueous hydrochloric acid for 20 minutes caused the loss of the pectin from the cell walls, and, consequently no red colouration or a very weak colour occurred after staining with ruthenium red, in sections of both resistant varieties. This result did not differ from that obtained with sections of cotyledons from susceptible seedlings, whether these had been treated with G 33 or not, as long as the cotyledons were free of the fungus.

In the scratched cotyledons the cells in a zone around the wound did not loose the capacity to take the red colour of ruthenium red, after previous treatment with hot 2% hydrochloric acid. This zone was found in inoculated cotyledons as well as in cotyledons which were free from hyphae. This indicated that the scratching of the cotyledons had changed the chemical or physical properties of the pectin of the cell walls in a zone surrounding the resulting wounds, whether hyphae were present or not. This result was different, however, from that obtained by the staining of sections from cotyledons of the susceptible variety. In the latter it was found, that changes in the chemical or the physical properties of the pectin of the cell walls in a zone around the wound, occurred only if the fungus was present, and that these changes were not induced by the mechanical damage caused by the scratching.

It is evident, therefore, that in the susceptible variety the walls of the intact cells bordering the scratch, react in a different way to mechanical damage as well as to the presence of the fungus, than do the cell walls of the two resistant varieties (table 1, see page 19).

Cellulose. The cell walls of the two resistant varieties normally stained blue with zinc chloride iodine, except in "Proso" with some cells in the zone that seemed to form a barrier against the advance of the hyphae from a wound.

Here the cell walls had a yellow colour,which was not changed after application of the reagent. This indicates a chemical alteration in their cellulose nature, which is probably associated with the formation of the yellow granular substance within and between the cells.

Also the cell walls of some cells in the neighbourhood of the uninoculated scratches, did not stain blue with zinc chloride iodine.

TABLE 1. Colour of the cell walls after treatment with hot 2% hydrochloric acid and subsequent staining with ruthenium red in cotyledons of cucumber, the surface of which was damaged by scratching

	Scratch inoculated with *Cladosporium*	Scratch not inoculated
Susceptible variety "Lange gele tros"	red zone around the wound	uncoloured
Resistant varieties "Mabro" and "Proso"	red zone around the wound	red zone around the wound

D. DISCUSSION

The two scab-resistant varieties under investigation, proved microscopically to be highly resistant. After normal spore germination and formation of appressoria, penetration of the intact surface of the host cotyledon tissue rarely occurred. The most successful case in that respect was found in "Mabro" where a hypha reached a point beyond the first layer of the palisade cells (fig. 11).

PIERSON and WALKER (53) reported in their study of the resistant variety "Maine No. 2", that penetration frequently took place, but that, owing to cell wall thickening and necrosis of some of the cells in the vicinity of the fungus, the disease was confined to a relatively small number of host cells. Such a mechanism was not found in the present study.

The damaged surface of the host tissue, if inoculated, was successfully penetrated by the fungus. As the hyphae passed from the region of dead cells to the intact living tissue their growth was impeded in a characteristic way in each variety. In "Mabro", the growth of the hyphae in the healthy tissue was checked at different distances from the damaged region, without any visible structural or chemical changes in the host cells or cell walls. In "Proso", resistance to invading hyphae was always linked to the formation of a yellow granular substance. Whether this substance is the cause of the resistance, or whether it is, just as the wound cork in other plants, merely a reaction to infection, remains open to further investigation.

The fact that the two resistant varieties "Mabro" and "Proso" were obtained from crosses between the same resistant variety "Highmoor", and two different susceptible varieties "Lentse gele" and "Spiers" respectively, is remarkable, since they differ as already shown above in the mechanism of checking the hyphae after successful penetration through wounds. This difference may be due to a different influence of each of the two non-resistant parents, upon the effect of genes responsible for resistance.

PIERSON and WALKER (53) found no gum formation in the resistant tissues of the variety "Maine No. 2". In the present investigation the yellow granular substance, which appeared to form the barrier to the fungus in the inoculated scratches in cotyledons of "Proso", stained with ruthenium red, and reacted

positively with phloroglucin-hydrochloric acid. In this barrier, the change in some cell walls proved to be due to their swelling which was associated with change in their chemical composition. This was more or less like the case of cell wall thickening in tissues of susceptible plants incubated at 27 °C as indicated by PIERSON and WALKER (53). The deposition of cell wall material which was recognised by the same investigators in tissues of resistant plants, was not found in the present work.

In the resistant variety, as in the susceptible seedlings that had been made resistant by treatment with G 33, the failure of the hyphae to grow indefinitely into the healthy cotyledon tissue is not due to contact with the protoplasm of the host cells, since the fungus is merely intercellular.

In the recent study of the resistance of tomato plants to *Fusarium lycopersici*, GOTHOSKAR *et al.* (32) reach the conclusion that a very labile substance which is continuously secreted by the host cells, impedes the growth of the pathogen. A similar process may be the cause of the resistance of "Mabro" and "Proso" to *Cladosporium cucumerinum*. Alternatively it may be assumed, that a substance, necessary to the growth of the fungus, diffuses out of the cells of the susceptible variety, whereas in the resistant variety this substance is lacking or remains within the cells.

CHAPTER III

THE DIFFERENCE IN REACTION TO THE CAUSAL ORGANISM, BETWEEN THE SUSCEPTIBLE OLDER CUCUMBER PLANTS TREATED WITH G 33 AND THE UNTREATED CONTROLS

A. INTRODUCTION

The foregoing chapters dealt with anatomical investigations on: 1. Susceptibility of cucumber seedlings of the variety "Lange gele tros", to the disease caused by *Cladosporium cucumerinum;* 2. induced resistance by treating susceptible seedlings with G 33; 3. genetic resistance of seedlings of two scab-resistant varieties. In these investigations the cotyledons were the only parts that had been inoculated and subjected to a study of the progress of the disease and the mode of resistance in 2 and 3. In the following two chapters the same investigations are described with somewhat older plants, of which stems, leaves and petioles had been inoculated.

The present chapter deals with the effect of G 33 on older cucumber plants inoculated in the fourth or fifth leaf stage with *Cladosporium cucumerinum*. Here again the anatomy of the host-parasite relationship in the tissues of treated and non-treated plants was studied.

B. MATERIALS AND METHODS

Seeds of the susceptible variety "Lange gele tros" were sown in sterilized sand in a flat square pot. As soon as the seedlings had developed two cotyledons and a growing point, they were transplanted each to one pot containing steam-sterilized soil. When the plants had developed 2–3 full-grown leaves and 2 younger ones, they were used for the experiments. In each experiment 20 plants were involved, of which 10 were treated with G 33. The compound was applied by daily adding of 15 ml. of a 500 p.p.m. solution to the soil of each pot for 3

successive days. On the fourth day treated and non-treated plants were inoculated. The two methods of inoculation previously described were applied, and for each method 5 treated and 5 untreated plants were used. The inoculated plants were placed in glass boxes; the plants sprayed with a spore suspension in one box, those inoculated by introducing spores in scratches on the leaf, stem and petiole surfaces in another. The plants were covered with thin sheets of polyethelene plastic for three days after inoculation. These sheets and also the constant presence of a thin layer of water on the bottom of the tin trays on which the pots were standing, helped much in the maintenance of a high humidity required for a successful infection. The temperature of the greenhouse in which the glass boxes were placed was constant at about 20 °C.

Samples of tissues from leaves, stems and petioles were daily collected, starting 48 hours after inoculation. The technique of fixation, sectioning and staining has been described in a previous chapter.

C. RESULTS

1. *Non-treated plants in relation to the pathogen*

a. Infection in leaves. Full-grown leaves were much more resistant than the still growing leaves. The latter showed well-developed external disease symptoms, i.e. irregularly distributed yellow lesions which later became brown in colour, whereas the former showed none of these symptoms.

The pathogen in immature leaves. Spore germination, formation of appressoria and the mode of penetration of the leaf surface were similar to the corresponding processes which had been investigated in cotyledons (chapter I).

The hyphae are intercellular as long as the host cells are alive, and grow only intracellularly when these cells collapse and become necrotic. In an advanced stage of infection (3–5 days after inoculation) the presence of the hyphae in the leaf tissue results in the secretion of two structurally different substances; viz. 1. a green or yellowish green, non-granulated substance; 2. a yellow granulated substance. The first substance fills entirely or partly many of the intercellular spaces of the infected tissue. The second substance is found mostly inside the cells, probably secreted by the affected protoplasm. The walls of these cells become yellow in colour.

The upper and the lower epidermis show the secretion of the granular yellow substance more than other tissues of the leaf. The palisade cells secrete both substances, the yellow substance being sometimes found between the cells. The non-granulated substance is mostly confined to the intercellulars of the spongy mesophyll, although a few cells of this tissue may secrete the other substance in their interior or intercellulars.

The vascular bundles are also affected, the walls of the vessels becoming deeply yellow in colour and occasionally one of the two substances is found between xylem or phloem parenchyma cells.

The highly refractive irregular rings or ellipses which appeared in the lumen of the palisade cells, and therefore constituted a striking feature of the diseased cotyledon tissue (chapter I), were found likewise in the lumen of the corresponding cells of infected leaf tissue.

Eventually (5–7 days after inoculation) the infected cells collapse and severe necrosis occurs, and the diseased areas become thin and somewhat transparent. Numerous conidiophores emerge from both the upper and lower epidermis. In

this respect the cotyledon differs from the leaf in that the conidiophores emerge in a smaller amount from the lower epidermis, which is often less damaged (chapter I).

It has been described in chapter I, that in infected cotyledons a yellow non-granulated substance occasionally occurs in the intercellulars of the mesophyll, or in the interior of its cells in very small amounts. In the infected leaf the abundance of the secreted substances is a conspicuous feature by which the infected leaf tissues differ from the infected cotyledon tissues. Moreover the secreted substance in the latter did not stain with either ruthenium red or with zinc chloride iodine, whereas the two different substances secreted in the leaf tissues stained with ruthenium red. In the different leaf sections there was much diversity as to the degree of staining with zinc chloride iodine of these secreted substances. Sometimes the latter coloured blue like cellulose, but in other sections they remained colourless. They stained a bright green in 0.002% of methyl green, but they did not stain with either, 0.1% of acid or basic fuchsin.

Most of the walls of infected cells which had become yellow did not give a cellulose reaction with zinc chloride iodine. These walls stained in the same way with methyl green as did the granular yellow substance, and, they likewise failed to stain in fuchsin solutions. As far as these two dyes are concerned the present investigation confirms the results of BEHR (8), on the staining of the brown granulated cell walls and the brown granules in the protoplasm of cells of the infected fruit. However, Behr reports that these cell walls stained red with phloroglucine-hydrochloric acid, whereas, in the present work the yellow walls of infected leaf cells did not give a reaction with this reagent.

The yellow walls of infected xylem vessels failed to give a reaction with phloroglucine-hydrochloric acid, indicating a chemical change in their lignified nature.

PIERSON and WALKER (53) reported in their study on the relation of the pathogen to leaf tissue in the susceptible variety "National Pickling", that small gum pockets had formed in the spongy mesophyll, and that larger ones, 10 to 12 cells in length, and 2 to 3 cells in width, had been formed in the softer tissues of the vascular bundles. This was not found in the present investigation.

The secretion of the granulated and non-granulated substances in the diseased leaf tissue, is not visibly connected with a breaking-down of the host cell walls.

The vessels in the vascular bundles remained free from hyphae throughout the course of infection.

In all other instances, e.g. the rate of spreading of the hyphae, etc. there was a complete resemblance between infected leaf and cotyledon tissues.

In the leaves, the epidermis of which was scratched, and a spore suspension was placed in the resulting scratches, the disease developed in the same way as described above.

The pathogen in mature leaves. Normal spore germination and development of appressoria took place, but no subsequent penetration by infection hyphae followed, not even through the dead cells in the scratches. This proves that mature leaves are highly resistant to infection by *Cladosporium cucumerinum*, and that this resistance is not exclusively due to the cuticle.

It is worthy noting that there was no visible microscopic difference in thickness of the cuticle between young and old leaf tissues.

b. Infection in stems. The younger internodes below the shoot apex were

heavily infected and showed as disease symptoms, elongated yellow spots later turning brown, whereas the older the internodes the more their tissues became resistant to the parasite.

The pathogen in young internodes. The spores germinated normally and formed well-developed appressoria. Direct penetration between two adjacent epidermal cells occurred within 48 to 72 hours after inoculation.

After passing the epidermis, the hyphae remained intercellular as long as the host cells looked healthy. The hyphae partly filled the intercellular spaces and at this stage, were distinctly thin.

As infection proceeded the walls of the diseased cells became yellow in colour and many of these cells and their intercellulars were filled with a granular yellow substance. This phase of infection appeared in all the different stem tissues successively, starting from the epidermis until it could be observed, 5–7 days after inoculation, in the inner phloem of the bicollateral bundles.

The yellow walls and the granular substance stained with ruthenium red, but did not stain with zinc chloride iodine, indicating a chemical change of the cellulose of the cell walls. They stained red with phloroglucin-hydrochloric acid but only locally. Sometimes only the thickenings at the corners of collenchyma cells, and the parts of the cell walls which border the triangular intercellular spaces between the parenchyma cells of the cortex, showed lignin reaction. Moreover, the walls of the diseased cells and the granular substance stained a bright green with 0.002 % of methyl green, while they showed no colouration with 0.1 % acid or basic fuchsin in water.

The same stage of infection is distinctly connected with the intracellular growth of the hyphae. The latter, which are now thicker than in the intercellular stage, spread without branching through the host cells, their tips occasionally swel as they touch the uncollapsed cell walls (fig. 16). BEHR (8) states, that during the intracellular habit of the mycelium, the hyphae form appressoria before leaving the cells or penetrating into them. In the present investigation, the swelling of the tips of some of the intracellular hyphae corresponds to what BEHR calls appressoria. It was clear that the occurrence of this swelling was not a rule.

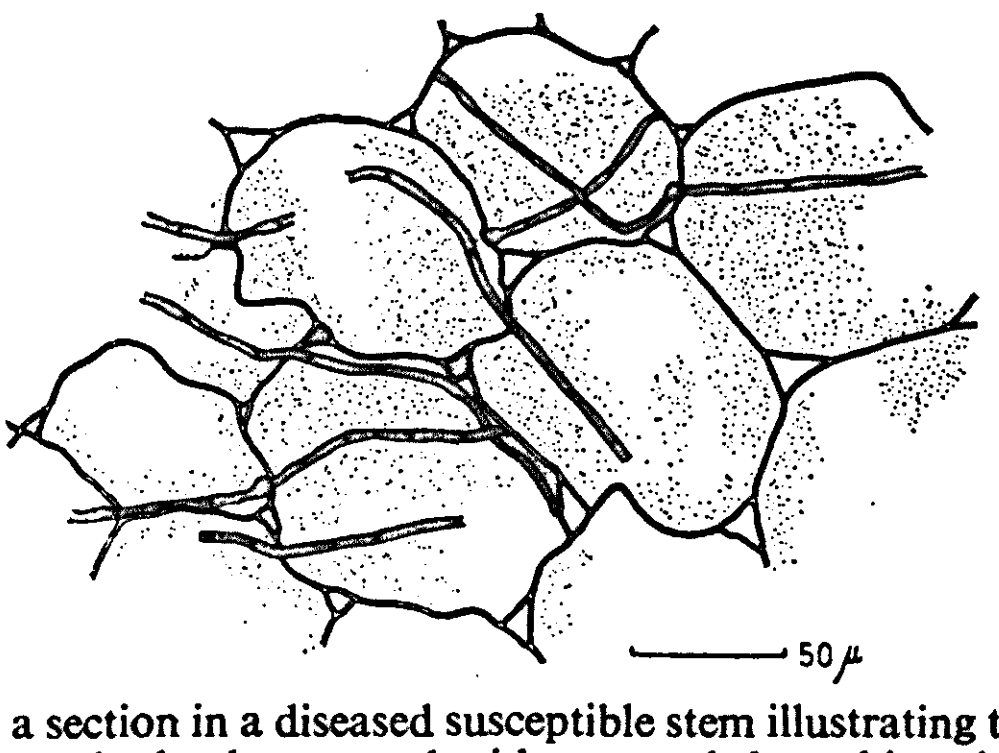


FIG. 16. A part from a section in a diseased susceptible stem illustrating the intracellular habit of the fungus. The hyphae spread without much branching through the host cells, their tips occasionally swell as they touch the uncollapsed cell walls. This intercellular habit which starts earlier than that in cotyledon or leaf is connected with the secretion by the infected cells of a granular yellow substance.

In the stem tissues, the intracellular habit of the hyphae seemed to start earlier

than in the leaf (or cotyledon) tissues, since in the latter, the fungus was not intracellular before the complete collapse and necrosis of the infected cells. PIERSON and WALKER (53) state that 120 hours after inoculation the protoplasm of many of the cells of the invaded stem tissues of the susceptible variety "National Pickling", had shrunken away from the cell walls, and that by this time, the fungus was generally growing intracellularly. This was not discovered in the present study, where a rapid transition from intercellular to intracellular growth apparently took place. Generally, the number of cell layers in which the fungus grew intercellularly was not more than $^1/_8$ to $^1/_{10}$ of the total number of the invaded layers, at 7 days after inoculation.

According to BEHR (8), the hyphae in the fruit tissues, which are infested but not yet destroyed, are exclusively intercellular. They become intracellular when invading the brown diseased cells.

Lysigenous cavities, reported by PIERSON and WALKER (53) to be a constant feature of their diseased stems, were found only occasionally. These cavities were only present in the parenchyma of the central cylinder; they were never found in the cortex, as was described by PIERSON and WALKER. Some of the lysigenous cavities were connected with a vascular bundle, but this was not always the case.

The cavities were formed out of several parenchyma cells, and occasionally contained hyphae (Plate 1: foto 2). They occurred in an advanced stage of the infection, after the fungus had started to grow intracellularly. They were filled with a non-granulated yellow substance. This substance stained with ruthenium red, indicating its probable pectin-like character. It stained also with 0.1% acid or basic fuchsin and with 0.002% of methyl green, but failed to react with phloroglucin-hydrochloric acid, and with zinc chloride iodine. BEHR (8) stained his granular brownish substance with fuchsin and methyl green only.

The central cylinder of both stems and petioles, contain strands of elongated thin-walled cells, filled with a dense protoplasm. Most of these strands are situated close to the perivascular fibres, and on the outer edge of the external and on the inner edge of the internal phloem. They run mainly in a longitudinal direction, but they are connected by strands which run more or less horizontally (Plate I: fotos 3 and 4). The latter are often branched and sinuous. In cross sections the strands appear as 3–5 angled cells, often associated with one or more small cells. In both, cross and longitudinal sections, they give the impression of sieve tubes with their companion cells. These strands which are common in the family of the *Cucurbitaceae*, have been described by FISCHER (29), who considered them to be sieve tubes. This author distinguished between vascular sieve tubes (i.e. the phloem in the vascular bundles) and peripheral sieve tubes (i.e. the strands outside the vascular bundles which run in a longitudinal direction). The connecting strands were called by Fischer "siebröhrencommisuren". Also ESAU (28) speaks of the strands in question as sieve tubes.

In the present paper these strands will be referred to as extra-fascicular "phloem". It will be shown below, that in diseased tissues, these strands behave differently from the phloem in the bundles.

In older stem (or petiole) tissues a considerable number of the extra-fascicular "phloem" elements become filled with a homogeneous substance (Plate I: foto 3). This substance stains with ruthenium red, sudan III and with acid and basic fuchsin. Moreover, it colours bluish with zinc chloride iodine; it reacts with phloroglucin-hydrochloric acid, and stains blue with the resorcin blue after

TSWETT. It behaves therefore, as a mixture of pectin, cellulose, callose and fatty substances.

In young and healthy internodes, the elements of the strands are quite free from this substance (Plate I: foto 4), but in corresponding diseased internodes they are filled with it (Plate I: foto 5). This indicates that an earlier and abundant accumulation of the substance is induced by the presence of the pathogen in the tissue.

The fact that only the extra-fascicular "phloem" elements, react in this way on infection, implies that they are physiologically different from the surrounding cells, and especially from the common sieve tubes which they resemble so closely morphologically.

Probably the extra-fascicular "phloem" has the capacity to store an excess of nutrients from other cells in the form of the above mentioned substance. Normally, in young tissues, the nutrients will be used up in the growth of the cells. If, however, the cells are damaged by the fungus, nutrient substances will leach out and may then be stored by the elements of the extra-fascicular "phloem".

An attempt was made by the writer to discover whether supplying the cucumber plants artificially with an excess of a nutrient material, would result in the filling of the extra-fascicular "phloem" elements, in the young stem or petiole tissues, with a substance similar to that described above. Ten plants in the sixth or the seventh leaf stage were heavily sprayed once a day with a solution of 5% sucrose for 7 successive days. In each spray, the whole plant was made thoroughly wet. The sucrose solution contained sulphanilamide at the concentration of 0.1% to prevent any possible contamination. Another ten plants in the same stage of development were set up as controls. They were sprayed with a solution of 0.1% sulphanilamide in distilled water.

Pieces from young and old stems, and others from petioles, were fixed at the end of the seventh day from plants sprayed with the sucrose solution and from the controls. Free hand sections were made and the extra-fascicular "phloem" elements were examined for the presence of the substance in question.

In the young stems and petioles these elements showed no accumulation of any substance in both treatments. In the older tissues there was no difference between the plants sprayed with the sucrose solution and their controls. In both cases only some of the elements were filled with the mentioned substance.

The only marked difference, was that spraying the plants with the sucrose solution causes them to grow somewhat faster than the control plants. This implies that supplying cucumber plants artificially with an excess of carbohydrates promotes the growth of these plants. This fact of growth response to applied sucrose has been previously reported by WENT *et al.* (75) with tomato plants, and by VAN KOOT *et al.* (70) with cucumber plants.

It is remarkable that the substance present in the lysigenous cavities differs from that in the elements of the extra-fascicular "phloem". The former did not react with zinc chloride iodine or with phloroglucin-hydrochloric acid, whereas the substance in the extra-fascicular "phloem" did not stain with 0.002% of methyl green.

The extra-fascicular "phloem" elements of the young diseased internodes, which were filled with the homogeneous substance indicated above, remained intact even when the surrounding cells had entirely collapsed.

The reactions to different reagents and the staining in different dyes of the

three substances described above; viz. 1. the granular substance in the interior of diseased cells; 2. the non-granulated substance of the lysigenous cavities; and 3. the non-granulated substance in the extra-fascicular "phloem" are summarized in table 2.

TABLE 2. The staining reactions of the different substances occurring in diseased cucumber tissues

Reagent or dye	Granular yellow substance	Non-granular substance from the cavities	Non-granular substance in the extra-fascicular "phloem"
Zinc chloride iodine	×	—	+
Acid or fuchsin 0.1 % Basic	—	+	+
Methyl green 0.002 %	+	+	—
Phloroglucin-hydrochloric acid	×	—	+
Ruthenium red	+	+	+
Resorcin blue	—	—	+

+ = positive reaction
— = negative reaction
× = sometimes positive, sometimes negative reaction.

The table shows that the three substances differ in their chemical composition. BEHR (8) stated that the substance in the lysigenous cavities was a true wound-gum after TEMME (66). The latter defined wound-gum as a substance which is formed by liquefaction of whole cells or tissues.

As to the presence of hyphae in the vessels, PIERSON and WALKER (53) stated that hyphae were occasionally found within the lumina of the large vessels. BOND (11) and SCHULTZ (61) reported that the hyphae became intracellular in the vessels. BEHR (8) on the other hand, mentioned that the vessels were free from hyphae. In the present investigation, hyphae were present in large and small vessels only when these vessels were surrounded by severely affected cells.

It was evident that the advance of the hyphae in the stem was slower than in the cotyledon and the leaf. This is possibly due to the presence of supporting tissues; viz. collenchyma and the perivascular fibres.

In young stems that had been inoculated by depositing the inoculum in scratches the mode of infection was similar to that described above.

The pathogen in older internodes. In most cases studied no penetration occurred despite normal spore germination and development of normal appressoria. Only in a few cases penetration took place, and even then infection did usually not proceed beyond the collenchyma of the cortex. In the most successful instances, a few parenchyma cells inside the collenchyma area were invaded. The invaded cells showed yellow walls and contained a yellow granular substance. Six to seven days after inoculation, after which time in younger

internodes big areas were severely infected and contained a great number of intracellular hyphae, the older internodes showed small infected spots with only very few intercellular hyphae. Just beneath these infected spots the underlying cells had divided by tangential walls to form a cork cambium.

The cuticle of the old stem did not differ in thickness from that of the young stem.

In the scratched older stems penetration occurred only through the dead cells of the scratches. The latter were surrounded by a cork cambium.

It is therefore, quite probable that a certain principle of unknown nature, is secreted by the host as it ages, and renders the whole tissue in question highly resistant to invasion by the pathogen.

c. Infection in petioles. The pathogen was capable of penetration in and of successful infection of the petioles of immature leaves, while the petioles of full-grown leaves were highly resistant.

Penetration and invasion of the petioles both young and old through the intact epidermis or through a wound, were similar to these processes in young and old stems respectively.

2. *Treated plants in relation to the pathogen*

The immature leaves, young stems and petioles of plants, which prior to inoculation, had been treated by adding daily to the soil of each pot 15 ml. of G 33 solution for 3 successive days, were much less diseased as compared with the severe infection of non-treated plants.

a. Infection in treated immature leaves. There was no effect of the treatment on the germination of spores and on the formation of appressoria, either on the intact surface or in wounds caused by scratching the upper epidermis. The appressoria, however, failed to develop any hyphae as long as the surface was intact. In the wounds made by scratching the epidermis hyphae developed from the appressoria and grew into the dead cells bordering the wounds, but rarely penetrated the adjacent healthy-looking cells.

b. Infection in treated young stems and petioles. In the majority of investigated cases no penetration occurred after germination of the spores and formation of appressoria.

In the few instances where penetration took place, the infection was restricted to a few layers of the cortex, even after 7 days from inoculation (Plate 1 : foto 6). The walls of the infected cells became yellow in colour, but complete necrosis and collapse of these cells never occurred. Moreover in the rare cases in which hyphae were visible, these were always intercellular. The whole infected area was surrounded by actively dividing cells which formed a wound tissue (Plate I : foto 6 and Plate II : foto 7).

It was evident, that the growth of the fungus in the treated stem or petiole tissues was slower than in the non-treated tissues, and therefore the healthy cells around the infected area could form a wound tissue before being invaded by the fungus.

In inoculated wounds made by scratching the surface, appressoria developed and formed hyphae which penetrated between the dead cells of the wounds. They rarely penetrated the adjacent microscopically healthy cells, which formed a cork cambium.

In mature and immature stems, petioles and leaves, the dead cells bordering a wound stained red with phloroglucin-hydrochloric acid. This phenomenon was independent of the presence of the fungus and occurred in plants treated with G 33 as well as in non-treated controls.

3. *Microchemical reactions*

Some microchemical reactions were carried out to investigate the effect of G 33 on the chemical composition of the cell walls in leaf tissues, and the nature of the secreted substances which on infection occurred abundantly in these tissues.

Pectic substances. Sections from healthy young leaves lost the pectin of the cell walls on heating in 2% hydrochloric acid for 20 minutes. Cell walls treated in this way gave no reaction with ruthenium red. The corresponding sections from plants treated with G 33 did not differ in this respect. This was also found with cotyledons (chapter I).

If, however, diseased leaves were treated with the hot acid, the capacity of the cell walls to stain with ruthenium red remained unchanged. In contrast to diseased cotyledons, which after treatment with hydrochloric acid stained in patches, the diseased leaves stained uniformly red.

In old leaves where the fungus does not penetrate, pectin of the cell walls remains soluble in the acid. This reconfirms that the insolubility in infected leaves is directly related to the presence of the fungus in the host tissue.

Cellulose. In an advanced stage of the disease (3–5 days after inoculation) most of the cell walls of upper and lower epidermis, and of many palisade cells showed a chemical change in their cellulose nature. They lost the capacity to stain blue with zinc chloride iodine. This was not found in diseased cotyledons.

There was no effect of G 33, upon the cellulose of the cell walls in leaves of treated plants.

Lignin. BEHR (8) reported that if a wound was made in a cucumber petiole with a scalpel, the walls of the dead cells along the wound surface and of several layers of healthy cells, stained red in phloroglucin-hydrochloric acid. In the present investigation, the healthy cells next to the wound made by scratching the surface of any part of the susceptible plant, never gave the phloroglucin reaction, whether this part was treated with G 33 or not.

"Gum". The abundant substances secreted in the interior of the cells and in many intercellulars of the diseased leaf tissues, always stained with ruthenium red and sometimes with zinc chloride iodine. The small amounts of the yellow substance secreted in the diseased cotyledon tissues, never stained with these two reagents (chapter I).

D. DISCUSSION

Tissues of cucumber plants show different liability to attack by *Cladosporium cucumerinum*, according to their age and stage of development. Thus the tips, including immature leaves, young internodes and petioles are severely damaged when inoculated with the fungus, whereas the corresponding older parts are much more resistant.

It was proved that the cuticle plays no role in this resistance, which may probably be due to some unknown principle related to host metabolism.

PIERSON and WALKER (53) in their histological work on susceptibility in the

variety "National Pickling" do not mention any resistance of this kind.

The present histological work reveals several similarities and differences between the different tissues of the host, regarding the microscopic symptoms and the course of the invading hyphae. On the one hand, diseased leaf and cotyledon tissues, differ as to the abundance of the substances secreted within and between the infected cells, the yellowish colour of the cell walls, and the degree of destruction of the upper and lower epidermis. On the other hand, the growth habit of the pathogen in both diseased tissues is the same, viz. intercellular as long as the host cells look healthy or are still intact; not until the complete collapse and necrosis of the cells do the hyphae start to grow intracellularly. According to PIERSON and WALKER (53) in the susceptible variety "National Pickling", intracellular hyphae develop in infected leaves, after 96 hours from inoculation, independent of the collapse of the tissue. Likewise both infected leaf and cotyledon tissues, show in the lumina of the palisade cells the rings and ellipses, which mark the circumference of the contact surface between the walls of two adjacent palisade cells. Probably outside these structures the middle lamella has been dissolved by a pectolytic enzyme from the intruding fungus.

In diseased stem and petiole tissues the growth habit of the pathogen changes earlier from inter- to intracellular, than in the leaf or in the cotyledon. This change in the growth of the fungus is connected with a particular stage of the infection. At this stage the walls of the diseased cells become yellow in colour, and many of these cells and of the intercellular spaces are filled with a yellow granulated substance. Thus, the intracellular growth of the hyphae does not start at the moment of the collapse and death of host cells, as in infected leaf and cotyledon tissues, but before that stage.

The effect of infection on the extra-fascicular "phloem" was not previously reported in literature. The name was coined by the author to indicate the strands of elongated thin-walled cells, filled with a dense protoplasm, which run outside the vascular bundles. The elements of these strands were considered by FISCHER (29) and ESAU (28) to be sieve tubes.

However, in young infected stems (or petioles), at an advanced stage of the infection, the mentioned elements are filled with a homogeneous substance, which if microchemically tested, reacts as a mixture of pectin, cellulose, callose and fatty substances. In young healthy stems (or petioles) these elements are entirely free from this substance.

As indicated above, it is probable that the damage of the invaded cells by the fungus, causes the nutrient substances to leach out, and may then be stored by the elements of the extra-fascicular "phloem".

The elements of the phloem in the bundles never contained the substance mentioned above. Therefore, it may be concluded that both kinds of phloem; viz. the normal and the extra-fascicular phloem, are physiologically different. In its function the latter resembles more the laticifers of other plants. In the present publication the name "phloem" is maintained because of the fact that FISCHER (29) found the tissue to contain sieve tubes. One may, however, ask whether it is right to use the same name for two tissues which are clearly different in function.

It was found, that infection had an effect on the pectin of the cell walls. Whereas the walls in healthy leaf tissues, after heating in 2 % hydrochloric acid failed to stain with ruthenium red, the walls of tissues containing the fungus retained the capacity of staining with this dye. Evidently the presence of the patho-

gen caused the pectin of the cell walls to become insoluble in acid. In the cotyledon, this change in the pectin is restricted to the areas occupied by hyphae. In the leaf, it also spreads to parts which do not yet contain the parasite. As stated in chapter I it seems as if a substance is diffusing from the fungus which changes the pectin of the cell walls, either by direct enzymatic action, or indirect by affecting the living cells. A quantity of such a substance enough to diffuse throughout the whole leaf, may be too small to reach all the parts of the much thicker cotyledon, and, hence in the latter may remain restricted to the neighbourhood of the infected areas.

On the intact cuticle of leaves from plants treated with G 33, infection hyphae did not grow from the well-developed appressoria. This was also found in cotyledons of the treated seedlings (chapter I). It may be concluded, then, that in the treated host, the cuticle acquires a resistance to penetration of the pathogen within the host.

If artificial wounds were inoculated, hyphae from well-developed appressoria grew out, but apparently remained restricted to the non-living cells of these wounds. In chapter I it is reported that through the corresponding wounds in treated cotyledons, the hyphae showed in addition, a small advance between the adjacent healthy-looking cells, but, they were soon checked from growing further.

However, after consideration of all these facts, it becomes quite clear that resistance induced by G 33 is located in the cuticle, as well as in the inner tissues of the host, and that the effect of G 33 does not extend beyond the cuticle itself to the exterior.

In internodes and petioles of the treated plants, similar results were obtained. In the few instances where small local lesions resulted it was clear that the growth of the pathogen was less than in tissues of the non-treated plants. Although the host cells could be affected to a considerable extent, the hyphae were merely intercellular even after one week from inoculation. This fact reconfirms that the effect of G 33 within the host tissue, does not depend on direct contact between the pathogen and the host protoplasm, but that an inhibitory principle might be present in the intercellulars and in the cell walls.

CHAPTER IV

THE RESISTANCE OF PLANTS OF THE VARIETIES "MABRO" AND "PROSO" AFTER THE SEEDLING STAGE

A. INTRODUCTION

In chapter II an anatomical study of the relationship between the cotyledons of the two scab-resistant varieties "Mabro" and "Proso", and the fungus *Cladosporium cucumerinum* was described.

The present chapter deals with the anatomical reactions to inoculation of other parts of these varieties.

B. MATERIALS AND METHODS

Plants of "Mabro" and "Proso" were raised from seeds until they had attained a stage similar to that described for the susceptible variety "Lange gele tros" (chapter III). Leaves, stems and petioles were inoculated in either of the two ways described above, and the plants were placed in an environment which was favourable to the development of the disease.

Material for investigation was collected daily. It was fixed and stained as described in previous chapters. The sections were made by hand.

C. RESULTS

1. *Infections in leaves, stems and petioles*

On both resistant varieties, "Mabro" and "Proso", the conidia of the fungus germinated and formed well-developed appressoria. No difference was observed in the way appressoria were formed by spores on the intact surface or by those applied in wounds made by scratching the host surface.

Through the intact host surface, however, penetration of the plant by hyphae formed by the appressoria rarely took place. In this respect there was no difference between the surface of leaves, stems and petioles, and that of cotyledons.

In the leaf tissue of "Mabro", only in one case, parts of a seemingly intercellular hypha were observed. This hypha did not extend far from the inoculated surface; its development had evidently ceased soon after it had passed beyond the first palisade layer (fig. 17). The section did not show any indication of the mode of entrance of this hypha through the epidermis. Two adjacent epidermal cells and three underlying palisade cells, all of them in the vicinity of the hypha were moderately affected. The protoplasm had secreted a yellow granular substance that filled the interior of the cells, but there was no complete necrosis or collapse of the latter. This very localized lesion was observed in a leaf that had been inoculated one week before. After the same period a susceptible leaf shows complete necrosis and collapse of the cells in large areas, where many hyphae have penetrated into the interior of the cells.

In the tissues of stems and petioles of "Mabro", a hypha from an appressorium had penetrated in a few cases (fig. 18). This figure which illustrates one of these

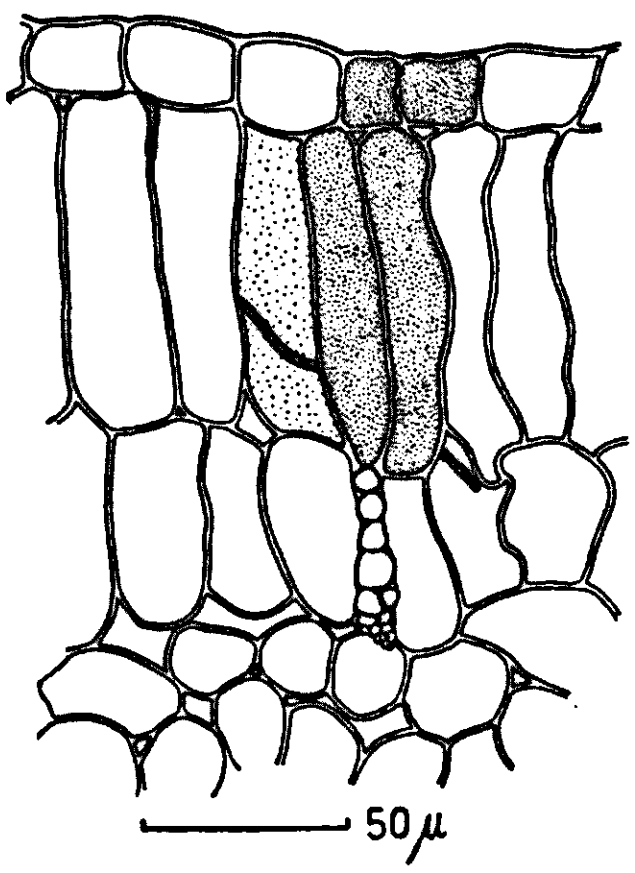


FIG. 17. The only penetration instance observed in leaf tissue of "Mabro". Parts of a seemingly intercellular hypha are observed. This hypha does not extend far from the inoculated surface, its development has evidently ceased soon after passing the first palisade layer. Few host cells in the vicinity of the hypha secrete a yellow granulated substance in their interior.

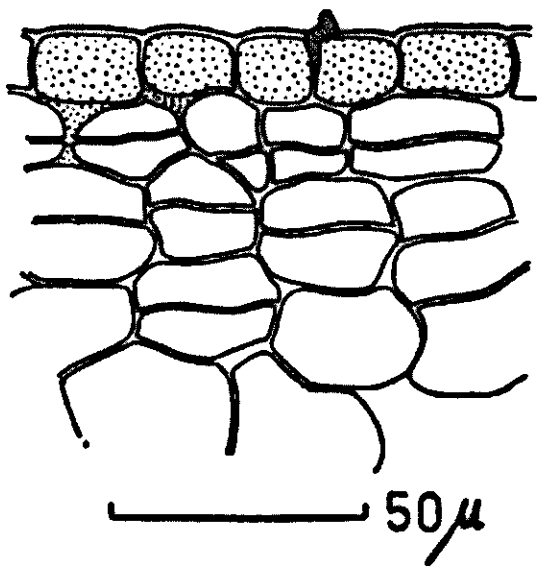


FIG. 18. One of the few cases where a very localized infection took place in stems and petioles of "Mabro". The hypha from an appressorium had penetrated between two adjacent epidermal cells. The adjacent epidermal cells were affected and secreted a yellow granulated substance in their interior and in a few of the underlying intercellulars. Cells of two to three collenchyma layers next to the epidermis showed division by tangential walls for the formation of a wound tissue. The latter was also formed around any artificial wound.

instances, is a cross section of an inoculated stem fixed 6 days after inoculation. The hypha had penetrated between two adjacent epidermal cells. It was difficult to follow its course further than the epidermal layer, but it was clear that only the adjacent epidermal cells had been visibly affected by the presence of the fungus. In these cells a yellow granular substance had been formed, whereas their walls showed a yellowish tint.

The cells in two or three layers of the underlying collenchyma had formed a number of new tangential walls, evidently the beginning of a protective wound tissue. Moreover, some of these collenchyma cells had lost, at their corners, the thickening of their walls, which is typical for the normal collenchyma cell.

The variety "Proso" was more resistant to penetration than "Mabro". Among all the preparations from different parts of "Proso" plants studied, only one was found in which an appressorium had succeeded in developing a hypha. This hypha had not proceeded further than the aperture of a stoma in the upper epidermis of a leaf. Here, neither necrosis nor collapse of cells in the vicinity, were visible.

In both varieties, there was no difference in resistance to scab between young and old leaves, stems and petioles.

If the surface had been damaged by scratching, the appressoria in the wound developed hyphae that penetrated between the dead cells bordering that wound. The hyphae rarely passed beyond the zone of the non-living cells to penetrate between the adjacent healthy cells. In the latter a cork cambium was formed around the wound. However, also around non-inoculated wounds a cork cambium was formed. These results were obtained with the different vegetative parts of the plants; viz. leaves, stems and petioles.

The differences in the way in which, in cotyledons of "Mabro" and in those of "Proso", the progress of the fungus was checked after inoculation of a wound (chapter II), were not found between leaves of these varieties. Here the pathogen was strictly limited to the dead cells of the wound. This indicates that wounded leaves are more resistant than wounded cotyledons.

Here again, the present results differ from those which PIERSON and WALKER (53) obtained with the resistant variety "Maine No. 2" in so far as in the latter the fungus frequently penetrated the tissues of leaves, stems and petioles. The arrest of the fungus by a reaction of the host which brings about a thickening of the cell walls, as described by these authors, was not found in the present investigation.

SCHULTZ (61) studied the reactions to the scab fungus of individual plants of the variety "Delikatesz", which showed different degrees of resistance. In susceptible plants the hyphae grew from the place of inoculation both intercellularly and intracellularly through the whole plant. The tissue showed no brown discoloration. In resistant plants there was a brown discoloration at the place of infection. At the edge of this brown zone a wound tissue of about 5–6 layers of cells was formed. In very resistant plants there were no hyphae in the neighbourhood of this cambium. In less resistant plants the hyphae penetrated the newly formed cells of the wound tissue. No wound tissue was formed in heavily infected plants. It was, however, not evident whether a causal relation existed between the formation of a wound tissue and the degree of resistance of the plant.

2. *Microchemical reactions*

Microchemical tests concerning the chemical composition of the cell walls of

the leaf tissue of both "Mabro" and "Proso", and the nature of the secreted yellow substance in affected cells in the sole case found, were carried out as noted above.

Pectic substances. The reactions to ruthenium red of the cell walls in leaf tissue of both varieties, were similar to those in the cotyledons (chapter II). Wounding the surface with or without subsequent inoculation gave the same result; viz. the formation of a zone of healthy cells around the wound, the cell walls of which retained their capacity to stain with ruthenium red, after pretreatment with hot 2 % hydrochloric acid.

Cellulose. The cell walls in leaves of the two resistant varieties, stained normally with zinc chloride iodine before and after inoculation with *Cladosporium*. Only in the preparation from "Mabro", in which a successful infection had resulted in a localized lesion (fig. 17), did the yellow walls of the affected cells not stain with zinc chloride iodine. This indicates a chemical change of their cellulose nature.

Lignin. If a wound in any vegetative part, of a "Mabro" or "Proso" plant was made by scratching of the surface, the dead cells around the wound gave a red colour with phloroglucin-hydrochloric acid after a few days. This reaction did not extend to the living cells. It occurred along wounds which had been inoculated as well as along uninoculated wounds.

"Gum". The secreted yellow substance, filling the interior of the affected cells in the single case observed in "Mabro" (fig. 17), reacted positively with ruthenium red, and slightly positive with zinc chloride iodine.

D. DISCUSSION

The microscopical study of the reaction to *Cladosporium cucumerinum* of older plants of the two varieties "Mabro ' and "Proso", proves that these varieties are highly resistant to scab-disease after the seedling stage, just as was found with the seedlings of the same varieties (chapter II).

On the intact surface of the different vegetative parts; i.e. leaves, stems and petioles; penetration of the cuticle rarely took place. This was also discovered with the cotyledons of seedlings (chapter II).

In the susceptible variety, the whole plant, as far as the vegetative parts are concerned, resists invasion by the fungus except at the tip which includes young and immature tissues. In the genetically resistant variety, the tip also resists the pathogen. Thus, under the conditions of the present investigation, the difference between the susceptible and the genetically resistant plant in reaction to the causal organism, is only found at the plant's tip. The tip of the genetically resistant plant is capable of producing an anti-infection principle, whereas in the former it is not. Once a resistance is induced to the tip of the susceptible plant, as in the present work by treatment with G 33, the whole plant becomes highly resistant.

In no vegetative part of the plant in both "Mabro" and "Proso", was the mechanism of resistance described by PIERSON and WALKER (53) of the variety "Maine No. 2", discovered. This implies that there is a varietal difference among the scab-resistant varieties, as to the mode of resistance to the causal organism. This is naturally controlled by genetic factors, but the prevailing environmental conditions may also play a part in this respect.

SUMMARY OF PART I

1. An anatomical study was carried out, to investigate the reaction to *Cladosporium cucumerinum* of susceptible cucumber tissues untreated and treated with G 33, and of tissues of two scab-resistant varieties.

2. Plants at the seedling stage and plants at the fourth to the fifth leaf stage were used in this study.

3. On the intact surface or in wounds in cotyledons of the seedlings, untreated and treated with G 33, and of the genetically resistant seedlings, the fungus spores germinate and develop well-defined appressoria.

4. Within 48 hours direct penetration freely took place through the intact surface of cotyledons of the susceptible seedlings untreated with G 33. Much less frequently stomatal penetration occurred. Inside the tissues the pathogen behaved as follows:

a. The hyphae grew intercellularly without much branching between the palisade cells, until they reached the spongy parenchyma where they spread more rapidly. The intracellular growth of the hyphae began only after the necrosis and collapse of the host cells.

b. During the intercellular habit of the fungus the hyphae were often in close contact with the cell walls; their tips being swollen when they struck the latter more or less perpendicularly.

c. In advanced stages of the infection, many of the palisade cells exhibit in their lumina highly refractive structures which appear as irregular rings or ellipses. These, when carefully studied, proved to mark the circumference of the contact surface between the walls of two adjacent palisade cells. It is suggested that the fungus secretes a pectolytic enzyme which dissolves parts of the middle lamella.

d. The hyphae while growing intercellularly did not cause any chemical change in the cellulose of the cell walls, even in parts of the walls with which they were in close contact.

e. The only striking effect of the hyphae on the chemical composition of the cell walls was that on their pectic material. If diseased sections were treated with hot 2 % hydrochloric acid for 20 minutes, the cell walls in parts occupied by the hyphae did not lose the capacity to stain with ruthenium red, whereas healthy sections lost this capacity after such a treatment. It is assumed that the pathogen secretes a substance which changes the chemical or the physical properties of the pectin of the cell walls, rendering it insoluble in acid.

f. "Gum" was present in the diseased tissues as a non-granulated yellow substance in very small amounts, within the cells or their intercellulars. This substance gave negative reactions with ruthenium red and zinc chloride iodine.

g. The hyphae crossed the distance between an inoculated upper epidermis and the lower epidermis within 3–6 days, depending on the prevailing environmental conditions.

h. In the final stage of the disease when the host cells were completely necrotic and collapsed, and the hyphae were intracellular, the conidiophores emerged abundantly through the remnants of the upper epidermis. They emerged in a smaller amount from the more intact lower epidermis, either through stomata or by direct penetration of the cuticle.

If artificial wounds in the cotyledons of untreated seedlings were inoculated the course of infection did not differ from that described above.

5. On the intact surface of cotyledons of the seedlings treated with G 33, penetration rarely took place. In a sole case observed, stomatal penetration occurred but apparently the penetrating hypha failed to proceed further than the aperture of the stoma.

If the surface was scratched and the resulting wounds were inoculated, the developing hyphae from the appressoria penetrated through the dead cells of the wound and between the adjacent healthy-looking cells, but they were soon checked to advance further than 1–2 cell layers beyond the damaged area.

There was no effect of G 33 on the pectin and cellulose constituents of the cell walls. The presence of the hyphae in small amounts within the inoculated scratches, caused the effect mentioned above on the pectin of the cell walls in a zone around these scratches.

6. In any of the older susceptible plants untreated with G 33, the tip which includes immature leaves, young internodes and petioles were heavily infected while the rest of the plant body was resistant under the conditions of the experiment.

7. In intact or scratched immature leaves, infection is established in an almost similar way to that in cotyledons. The only differences exist in the amount of substances secreted by the host protoplasm and their reaction to certain dyes, the chemical change in the cellulose nature of the cell walls, the degree of damage caused to the lower epidermis, and the extent of the effect of the fungus on the pectin of cell walls.

8. On the intact surface of the younger internodes (or petioles) normal spore germination and formation of appressoria take place. Direct penetration occurs within 48–72 hr. after inoculation. As the hyphae pass through the epidermis they show intercellular habit which is soon changed to an intracellular habit as the host cells exhibit a particular phase of infection. It seems, that the intracellular habit of the pathogen starts here earlier than in leaves or cotyledons. The tips of the intracellular hyphae occasionally swell as they touch the uncollapsed cells. In an advanced stage of infection and after the establishment of the intracellular growth of the fungus, lysigenous cavities are occasionally formed in the parenchyma of the central cylinder. The cavities are filled with a non-granular yellow substance. The extra-fascicular "phloem" is a name given by the author to strands of elongated thin-walled cells, filled with a dense protoplasm. These strands are well known in the *Cucurbitaceae* and are considered by other authors to be sieve tubes. On infection they are filled with a non-granular yellow substance, indicating that they are physiologically different from the normal sieve tubes which were free from this substance.

Microchemical tests, show that the three substances present in the diseased stem (or petiole) tissues; viz. the granular substance within the cells, the non-granular substance of the lysigenous cavities, and the non-granular substance in the extra-fascicular "phloem", differ in their chemical composition.

Hyphae were present in the vessels only when they were surrounded by severely infected cells.

Infection through wounds gave the same results as described above.

9. Old internodes (or petioles) were highly resistant to penetration, whether the intact surface was inoculated or the spores were deposited in scratches. The

thickness of the cuticle plays no role in this respect, but it is proved that resistance occurs in the cuticle as well as in the inner tissues.

10. On treating cucumber plants with G 33, their tips become resistant to the pathogen. On the intact surface of the different vegetative parts there was no penetration after the formation of apressoria. Only in a few cases in the stems and petioles, did the hyphae penetrate into the host, but their growth was slowed down and the infection was restricted to small localized lesions. Around the latter the adjacent healthy cells had time enough to form a cork tissue. In the inoculated scratches the developing hyphae were restricted only to the dead cells bordering these scratches.

11. From the results obtained by treating the seedlings or older plants with G 33, it is concluded that; *a.* the resistance induced by G 33 is located in the cuticle as well as in the inner tissues, *b.* the G 33 is only effective in cells which at least are microscopically healthy-looking, *c.* the resistance is not due to a plasmatic interaction, *d.* the effect of G 33 does not extend beyond the cuticle to the outer surface.

12. The two scab-resistant varieties "Mabro" and "Proso", proved microscopically to be highly resistant during as well as after the seedling stage.

On the intact surface of the cotyledons, leaves, stems and petioles the spores germinated readily and formed normal appressoria. The latter, however, failed to produce infection hyphae in the majority of cases studied. Only in a few cases were hyphae observed inside the host tissue but apparently they were impeded from growing further than the epidermis in stems and petioles; and in leaves and cotyledons, in the most successful cases they had soon stopped their development after passing the first palisade layer. The host cells in the vicinity of these hyphae may or may not be affected, but complete necrosis or collapse of these cells never occurred. A cork tissue is always formed in stems and petioles around the small infected lesion. This tissue occurs naturally around any wound in the host.

If the scratches made on the surface of any vegative part except the cotyledons, were inoculated, the hyphae which developed from appressoria grew only in the non-living cells, and very rarely between the adjacent healthy cells. The case in scratched cotyledons differs in that the hyphae invade the healthy cells but they fail to grow indefinitely owing to a mechanism which is possibly different in each of the two varieties.

The chemical composition of the cell walls in tissues of the resistant varieties does not differ from that of tissues of the susceptible variety.

Scratching the surface of the cotyledon or leaf, results in the appearance around the wounds of a zone of healthy cells, the walls of which react positively with ruthenium red after treatment with hot 2% HCl. This phenomenon is independent of the presence of the fungus.

13. In the genetically resistant plants, just as in the G 33-treated plants an inhibitory principle impedes the growth of the fungus. This principle is not present on the outer surface.

PART II

STUDIES ON THE NATURE OF GENETIC AND OF INDUCED RESISTANCE, TO CUCUMBER SCAB-DISEASE

From the results represented in the foregoing chapters, it follows that in the genetically resistant plants as well as in the G 33-treated plants a principle is present that inhibits the growth of *Cladosporium cucumerinum*. This principle is absent on the outer surface, for here the spores of the fungus germinate readily. It may be now asked, whether the suggested inhibiting principle is produced in both types of resistance by similar or by different mechanisms. In the following an attempt is made to answer this question.

CHAPTER V

1. THE INFLUENCE OF INHIBITORS OF PHOSPHORYLATION AND RESPIRATION ON THE GENETIC AND G 33-INDUCED RESISTANCE

A. INTRODUCTION

The effect of respiratory inhibitors on the resistance of tomato plants to wilt caused by *Fusarium lycopersici* has been very recently investigated by GOTHOSKAR *et al.* (32). Following the problem from its starting point, SCHEFFER and WALKER (59) in their work on distribution and nature of *Fusarinm* resistance in tomato plants, concluded that, although the relation of host metabolism to disease resistance is difficult to interpret, it is possible that resistance is dependent upon continuous metabolic activity. On considering this possibility, GOTHOSKAR *et al.* claimed that if resistance is related to metabolism, substances which affect metabolism should alter resistance.

They investigated the effect of 2,4 dinitrophenol (DNP) on respiration and on the resistance of tomato plants. At a concentration of 10^{-5}M, this compound caused an increase in oxygen uptake of 33% over the control. This indicates that at this concentration the DNP blocked the transfer of energy from respiration to synthetic processes. If applied to tomato plants, the same concentration of DNP strongly promoted the development of the disease.

GOTHOSKAR *et al.* then investigated the effect of specific respiratory inhibitors on resistance. Sodium fluoride was applied for 8 days, thiourea for 12 days, sodium diethyl dithiocarbamate for 6 days, sodium malonate and sodium fluoroacetate for 9 days. The first three compounds caused a breakdown of resistance. The authors proved that this breakdown of resistance, cannot be attributed to stimulation of growth of the pathogen. At the end of their work, they concluded that the results of their investigation strongly indicate that the resistance is closely bound up with the metabolism of the resistant host. When substances which are known to inhibit phosphorylation or certain stages in the respiratory cycle, are introduced into the transpiration stream of resistant plants, the fungus produces symptoms identical with those normally produced by natural infection of the susceptible host. They suggest, therefore, that in the resistant plant some substance is continuously being formed that accumulates tc a level where it becomes toxic to the fungus and blocks its advance. This

substance must either be very labile or continuously metabolized to nontoxic materials, since extracts of resistant plants are not toxic to the fungus.

In a previous chapter, the author suggested that a similar process may be the cause of the resistance of "Mabro" and "Proso" to *Cladosporium cucumerinum*. Therefore an attempt was made to investigate the influence of 2,4 dinitrophenol (DNP), as an inhibitor of phosphorylation, and of sodium diethyldithiocarbamate (NaDDC), and of sodium azide (NaAZ) as respiratory inhibitors, upon genetic resistance of the two scab-resistant varieties "Mabro" and "Proso". Moreover their effect on resistance induced by treatment with G 33 was studied.

B. EXPERIMENTAL RESULTS

1. *The effect of inhibitors of phosphorylation and respiration on genetic resistance*

Seedlings of "Mabro" and "Proso" when possessing a root system, a hypocotyl, two cotyledons and a growing point were used for the experiments.

Solutions of 2,4 dinitrophenol (DNP), sodium diethyldithiocarbamate (NaDDC) and sodium azide (NaAZ) at the concentrations of 10^{-3}, 10^{-4}, 10^{-5} molar, were tested for their toxicity to cucumber seedlings.

Groups of 4 seedlings from "Mabro" were placed each in a 50 ml. wide mouth bottle filled to a height of about 2 cm. with one of the concentrations mentioned above. Only the roots and the lower part of the hypocotyl were in the fluid. Seedlings of "Proso" were treated in the same manner. The bottles were left in the greenhouse for 2 days.

It was found that with both DNP and NaDDC the concentrations 10^{-3} molar and 10^{-4} molar were injurious to seedlings of "Mabro" and "Proso", while NaAZ was so at the concentration of 10^{-3} molar only. The toxicity was manifested by the extreme thinness of the hypocotyl or by the presence of local constrictions, or by softening of the root system which lost almost its firm appearance.

The concentrations used henceforth throughout the experiments were: 10^{-5} molar for both DNP and NaDDC; and 10^{-4}, 10^{-5} molar for NaAZ.

Solutions at these non-toxic concentrations were prepared. Two 50 ml. wide-mouth bottles were filled with each solution to a height of about 2 cm, and groups of "Mabro" seedlings were placed in these bottles as described above. The same treatment was applied to "Proso" seedlings. Untreated seedlings of the two varieties and seedlings of the susceptible variety "Lange gele tros", were set up as controls.

The bottles were left in the greenhouse for 48 hours, after which the treated seedlings and the controls were inoculated by spraying with an aqueous spore suspension. The bottles were then placed in glass boxes in another greenhouse where environmental conditions were made favourable for infection.

After a lapse of 7 days from inoculation, none of the untreated seedlings showed disease symptoms. Also the majority of the treated seedlings showed no symptoms of the disease. The susceptible seedlings, on the contrary, were heavily infected.

Only in seedlings of "Mabro" treated with DNP 10^{-5} molar, locally distributed faintly yellow spots developed on the cotyledon surface. Microscopical study of hand sections stained in cotton blue lactophenol, from these cotyledons, revealed successful penetration into the host tissue at some places. The growth of the hyphae inside the tissue was only intercellular (fig. 19); in some instances the mycelium reached the lower epidermis, in others not. The host cells were

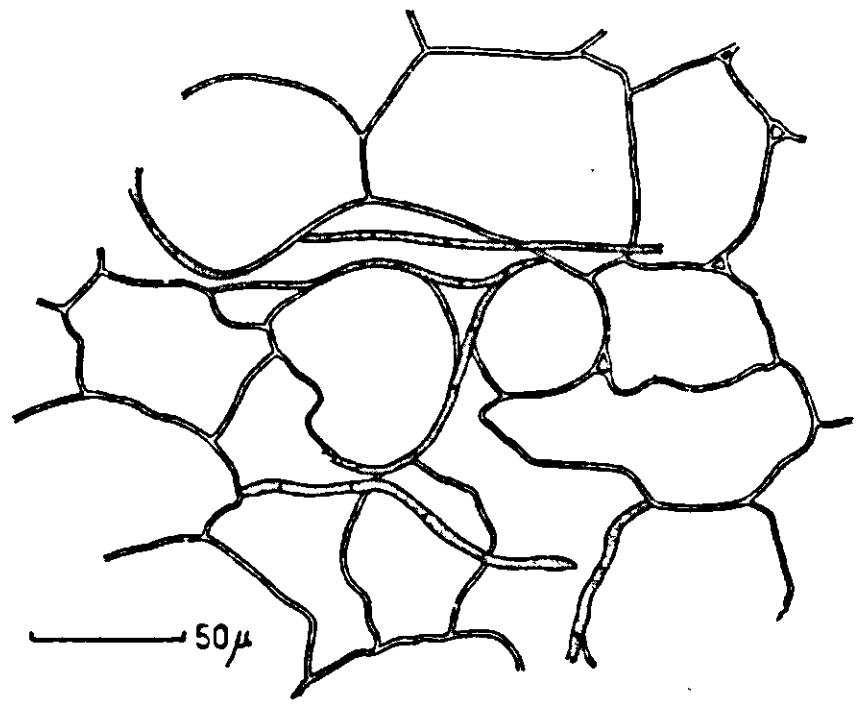


FIG. 19. A part from the spongy tissue of a cotyledon from a "Mabro" seedling treated with DNP 10^{-5}malar. This tissue which is genetically highly resistant to the pathogen became after treatment mildly infected. Although the cotyledon was fixed 8 days after inoculation the intruding hyphae were merely intercellular, and the host cells were neither necrotic nor collapsed.

mildly affected; their walls being slightly yellowish, but on the whole no collapse or necrosis of the host tissue took place. The host cells remained quite intact, even after 7 days from inoculation, the same period after which severe necrosis to the susceptible tissue occurred.

A second series of experiments gave similar results.

Therefore it was clear that the DNP which inhibits phosphorylation, and the two respiratory inhibitors NaDDC and NaAZ, had no influence upon the mechanism of resistance in tissues of "Mabro" and "Proso", the two scab-resistant varieties.

Even in the case of "Mabro", when seedlings treated with DNP 10^{-5} molar were mildly affected (the hyphae succeeded in penetrating and growing inside the host tissue to some extent), complete and normal infection failed.

2. *The effect of inhibitors of phosphorylation and respiration, on G 33-induced resistance*

The results concerning the toxic effect of the 3 inhibitors mentioned above, at different concentrations on tissues of the susceptible variety "Lange gele tros" were identical with those obtained with tissues of the resistant varieties "Mabro" and "Proso".

Seedlings of the susceptible variety treated with 100 p.p.m. G 33 solution during 2 days, in the same way as described in chapter I were placed in groups of 4 seedlings in eight 50 ml. wide-mouth bottles. Two of these bottles were filled to a height of about 2 cm. with one of the DNP, NaDDC or NaAZ non-toxic solutions. The seedlings were then inoculated by spraying with an aqueous spore suspension. Seedlings treated only with G 33, and normal susceptible seedlings were also inoculated and set up as controls.

The inoculated seedlings were kept in the greenhouse under appropriate conditions for scab-disease.

Still another method of treating the seedling was adopted. The seedlings were placed in wide-mouth bottles filled to a height of about 2 cm. with a mixture of G 33 solution and one of the non-toxic solutions in the proportion of 1:1. In this case double concentrations of G 33 and the inhibitors were used. Thus the experiment included: 2 bottles containing a mixture of (200 p.p.m. G 33 + 2 ×

10^{-5} molar DNP); 2 with a mixture of (200 p.p.m. G 33 + 2 × 10^{-5} molar NaDDC); 2 with a mixture of (200 p.p.m. + 2 × 10^{-4} molar NaAZ); and 2 with a mixture of (200 p.p.m. G 33 + 2 × 10^{-5} molar NaAZ). After placing a group of 4 seedlings in each of these bottles, they were left in the greenhouse for 48 hours, together with 2 bottles containing seedlings in a 100 p.p.m. G 33 solution, and 2 bottles containing seedlings in distilled water. At the end of the 48 hr., all the seedlings were transferred to other bottles containing fresh distilled water after rinsing the root system thoroughly under the tap. They were then sprayed with the inoculum, and placed in bottles in glass boxes under conditions favourable for scab.

In no case was there any effect of DNP, the phosphorylation inhibitor, or of NaDDC and NaAZ the respiratory inhibitors, on the resistance induced by treatment with G 33. Whether the seedlings were transferred to the inhibitor solution after treatment with G 33, then inoculated, or treated with a mixture of G 33 + inhibitor for 2 days, and then inoculated, had no influence on the results.

Repetition of the experiments gave similar results.

Foto 10, (plate II) shows one of the experiments where seedlings treated with G 33 were transferred to solutions of NaDDC and NaAZ, then inoculated and observed after a week, for the development of disease symptoms.

Foto 11 (plate III) illustrates at the end of one week after inoculation, the result with DNP when the other method for introducing the inhibitors was adopted.

If the susceptible seedlings were treated with the solutions of the inhibitors indicated above for 2 days and then transferred to distilled water, and inoculated, symptoms of disease developed normally as in natural infection in the non-treated seedlings. This indicates that there was no influence of these inhibitors, which are known to block phosphorylation or respiration, on the degree of susceptibility of the tissues of a susceptible host.

C. DISCUSSION

Except in one case, in which a slight infection of DNP-treated "Mabro" seedlings was observed, 2,4 dinitrophenol, sodium diethyldithiocarbamate and sodium azide had no influence on the genetic resistance of the plants to *Cladosporium cucumerinum*.

The same was found with G 33-treated seedlings. The 3 mentioned substances did not decrease the resistance which had been induced by the treatment with G 33.

It may be asked, whether they had an effect on cucumber tissues at the concentrations used in the present experiments. It was not attempted to answer this question in the present investigation. The concentrations used were fairly high, as the next higher concentration caused considerable visible damage to the plants. It will be shown in the following pages, that it is possible to break down the resistance of the plants to *Cladosporium* by treatment with chemicals, without damaging them permanently.

The mild infection caused to "Mabro" tissues when treated with DNP 10^{-5} molar, and the non-infection of "Proso" tissues after the same treatment, implies that difference in degree of response to such a phosphorylation inhibitor as DNP is varietal.

The local effect of DNP in "Mabro" tissues, may be attributed to an uneven distribution for one reason or the other, of the substance into the host tissue.

The results evidently showed that no difference can be shown between the mechanism of genetic resistance, and that of G 33-induced resistance by using inhibitors of phosphorylation or respiration as those applied in the present work.

Moreover, it can be concluded that the mechanism of resistance in tissues of "Mabro" and "Proso" to *Cladosporium cucumerinum* is different from that in tissues of the tomato variety "JEFFERSON" to *Fusarium lycopersici*. GOTHOSKAR *et al.* (32) concluded that treatment of "Jefferson" cuttings with respiratory inhibitors broke down resistance. In the present investigation such a conclusion was not reached.

2. THE INFLUENCE OF NARCOTICS ON THE GENETIC AND G 33-INDUCED RESISTANCE

A. INTRODUCTION

One of the aims of the experiments described in the present chapter was to determine whether narcotics influence genetic and induced resistance.

BEHR (9) carried out an investigation on the effect of some narcotics on the infection of resistant potato tubers by *Phytophthora infestans*. He found out that, on relatively resistant tubers which have been treated with chloroform at a concentration of 0.17 ml. per litre of air, the fungus grew better than on untreated resistant tubers. Not only was resistance against *Phytophthora infestans* decreased, but this was also the case against facultative parasites, which are not natural invaders of potato plants, such as *Fusarium solani*, etc.

It is supposed by BEHR that narcosis inhibits the formation of fungicidal protective substances in the plants.

MIZUKAMI (51) made a study on the resistance of rice seedlings treated with ether vapor to *Helminthosporium* disease. He found that rice seedlings treated with ether and then inoculated with the spore suspension of the fungus, had an increased number of leaf spots in comparison with the non-treated controls. He therefore concluded that the resistance of rice seedlings to *Ophiobolus miyabeanus* ITO et KURIBAYASHI was decreased, due to the treatment with ether vapor.

In the present investigation ether and chloroform were used as narcotics. These substances inhibit several processes in the cell in a non-specific way.

B. EXPERIMENTAL RESULTS

1. *The effect of narcotics on genetic resistance*

Seedlings of "Mabro" and "Proso" at the same stage as described above were used. After rinsing the roots to remove adhering sand grains, groups of 4 seedlings were placed in 50 ml. wide-mouth bottles filled with water to a height of about 2 cm. Two of these bottles from "Mabro" and two from "Proso" were carefully placed inside a one and a half litre bottle, which had been partly filled with 300 cc. of a 5% saturated ether solution. After the introduction of the seedlings, the large bottle was tightly closed. Only the lower half of the small bottles was immersed in the solution. The same was repeated for other and lower concentrations; namely, 2.5%, 2%, 1.5% and 1%.

Another experiment was carried out in which chloroform was used instead of ether. The solutions involved were at the concentrations of: 1% (saturated), 0.5%, and 0.25%.

The big bottles with their contents, were then kept in the greenhouse under the prevalent environmental conditions. After about 14 hours many of the solutions involved showed a toxic effect on the host seedlings. On exposure to an atmosphere of ether 5% or 2.5% or 2%, and of chloroform 1% or 0.5% for such a relatively short duration as 14 hr. they were severely injured. Extreme thinness of the hypocotyl, discoloration, and loss of turgidity of the cotyledons were the pronounced features.

Ether at 1.5% and 1% concentrations, and chloroform at the concentration of 0.25% had only a slight effect. The affected seedlings being slightly drooped.

These narcotised seedlings were then taken out of the small bottles, and placed in new ones containing fresh water. Half of the seedlings were inoculated by spraying with an aqueous spore suspension, the other half was left uninoculated in the open air to note whether recovery took place.

The inoculated seedlings were placed in a glass box under conditions favourable for the progress of scab-disease.

Non-narcotised seedlings of "Mabro" and "Proso", and seedlings of the susceptible variety were also inoculated and served as controls.

Two days after inoculation, in both "Mabro" and "Proso", a superficial and massive growth of the fungus was clearly visible on parts of the upper and the lower surfaces of the narcotised cotyledons.

Photo 8 (plate II) represents a hand cross section stained in cotton blue lactophenol, of a narcotised cotyledon of "Mabro", fixed two days after inoculation. The depth of the superficial mycelial growth is nearly equal to the cross section of the cotyledon itself.

Photo 9 (plate II) shows in higher magnification more details of this superficial growth, which is also associated with an abundant growth of the hyphae inside the host tissue.

It was very difficult for the writer to determine whether intercellular habit preceeded intracellular habit, or if the two took place simultaneously. After such a short period as two days from inoculation, the host cells were so concealed by the extraordinary growth of the pathogen, that it was impossible to study the interaction between the fungus and the invaded cells. It seems as if a part of the host nutrients diffused out of the narcotised cells, and reached the upper and the lower surfaces of the cotyledons. It is probable that because of this the massive superficial and internal growth of the fungus was greately stimulated.

THATCHER (67) worked on osmotic and permeability relations in parasitism, and found out that increase in host cell permeability by chloroform leads to an increase in susceptibility to rust. From THATCHER'S results it appears possible that the cell membranes of the narcotised cells, in the present investigation, become much more permeable than the non-narcotised cells.

The effect of ether or chloroform is reversible. Seedlings which have been treated with a certain concentration, are allowed to be for a few days in the atmosphere of the greenhouse. They regain their resistance to *Cladosporium* after that period. Upon inoculation such seedlings remain perfectly healthy, whereas seedlings which are inoculated immediately after treatment with the narcotics are destroyed by the fungus.

As described above the phosphorylation inhibitor and the two respiration inhibitors investigated have no influence upon the resistance of "Mabro" and "Proso". In other words if a substance is excreted by the genetically resistant

host which inhibits the fungus, this process is independent of respiration or of phosphorylation in the host.

With narcotics, the case is quite different, for metabolic processes other than respiration and phosphorylation are also inhibited by these substances. As a result of this inhibition the narcotised tissues of the two scab-resistant varieties completely lost the capacity to resist the pathogen. The normal relationship between susceptible tissues and the causal organism was entirely lacking. It was substituted by an abnormal growth of the mycelium over and inside the host tissue, shortly after inoculation, and the invaded cells could scarcely be detected.

Photo 12 (plate III) demonstrates one of the experiments to determine the effect of narcosis on genetic resistance. The bottles 1 and 2 show the complete recovery of "Mabro" seedlings narcotised with 0.25% chloroform, after exposure to the open air for 2 days and the severe infection of the narcotised seedlings, 2 days after inoculation. The bottles 3 and 4 represent seedlings of "Proso" treated in the same manner. Those numbered 5 and 6 for "Mabro" seedlings and 7 and 8 for "Proso" seedlings illustrate similar results when ether at the concentration of 1.5% was used.

The fungus failed to grow over or inside the narcotised tissues, when these were kept in a constant atmosphere of ether or chloroform after inoculation.

Repeated experiments confirmed the results described above.

The narcotised tissues of "Mabro" and "Proso" were inoculated with two facultative parasites which are by nature not parasitic on cucumber tissues; namely *Penicillium italicum* and the much less specialized fungus *Botrytis cinerea*. The aim was to note whether narcosis leads to infection by non-congenial fungi. The spores of the two fungi germinated, and developed germ tubes on the host surface. However, no penetration and no subsequent infection took place. This implies that the narcotised tissues remain resistant to fungi which by nature do not attack cucumber plants. Moreover, it is quite clear that the mechanism of the genetic resistance of cucumber tissues to *Cladosporium cucumerinum*, is different from that of the natural resistance of the same tissues to *Penicillium italicum* or *Botrytis cinerea*.

A quantitave experiment was carried out to demonstrate the interaction described above between the narcotised seedlings of "Mabro" and "Proso" and the three fungi *Cladosporium cucumerinum*, *Penicillium italicum* and *Botrytis cinerea*.

The seedlings used were those of "Mabro" and the narcotic applied was ether 1.5%.

150 seedlings of "Mabro" were raised, and with each of the three fungi 30 narcotised seedlings were inoculated. In the greenhouse thirty narcotised seedlings were left without inoculation for recovery. Thirty non-narcotised seedlings of "Mabro', and 30 susceptible seedlings of the variety "Lange gele tros" were inoculated with *Cladosporium cucumerinum* and served as controls. The inoculated seedlings were kept under favourable conditions for scab disease and for attack by *Penicillium italicum* and *Botrytis cinerea*.

Two days after inoculation more than 95% of the narcotised seedlings were severely infected by *Cladosporium cucumerinum*. After a week from inoculation all the narcotised seedlings inoculated with *Penicillium italicum*, and more than 90% of the narcotised seedlings inoculated with *Botrytis cinerea*, remained resistant to these two fungi not-adapted to cucumber plants. As expected the non-narcotised seedlings of "Mabro" were highly resistant to scab while the susceptible seedlings were badly infected.

From a total of 30 narcotised seedlings 28 seedlings recovered on exposure to the open air for 48 hr. and regained their normal appearance.

Table 3 shows the number of healthy and of diseased "Mabro" seedlings after having been narcotised and inoculated with the three fungi in question.

TABLE 3. The interaction between narcotised "Mabro" seedlings and *Cladosporium cucumerinum*, *Penicillium italicum*, and *Botrytis cinerea*

Number of healthy and diseased "Mabro" seedlings after being narcotised and then inoculated with:					
Cladosporium cucumerinum		*Penicillium italicum*		*Botrytis cinerea*	
Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
1	29	30	0	28	2

The result obtained from this experiment reconfirms the conclusion that the genetic resistance of cucumber plants to *Cladosporium cucumerinum* and the natural resistance of the same plants to *Penicillium italicum* and *Botrytis cinerea* are based on different mechanisms, since the first type of resistance was broken down by narcosis while the second type was not.

2. *The effect of narcotics on G 33-induced resistance*

It was found that the seedlings of the susceptible variety "Lange gele tros" can also endure narcosis by ether and chloroform for a period of 14 hr. at the concentrations of 1.5% and 0.25% respectively, without being injured or distorted. The only effect was that the cotyledons began to droop.

The narcotised seedlings recovered within 48 hr. when exposed to the open air.

Ten 50 ml. wide-mouth bottles, were filled to a height of about 2 cm. with a solution of 100 p.p.m. of G 33. In each bottle 4 seedlings were inserted and the bottles were left in the greenhouse. After 2 days, 4 of these bottles were placed in an atmosphere over a solution of ether 1.5% in water, and another four in an atmosphere over chloroform 0.25% in the same way as described with "Mabro" and "Proso" seedlings. The remaining two bottles remained on the bench in the greenhouse as controls.

After a lapse of 14 hr. the bottles were lifted out of the narcotic atmosphere. Then, the root systems of the seedlings were thoroughly washed under the tap, and the seedlings were replaced in fresh distilled water. Half of the narcotised seedlings, and all the non-narcotised seedlings were then inoculated with an aqueous spore suspension, and were kept under favourable conditions for scab disease. Seedlings not treated with G 33 were also inoculated and served as controls. The other half of the narcotised seedlings were exposed to the open air, to see whether these seedlings would recover from narcosis.

The narcotised and the non-narcotised seedlings treated with G 33 showed no difference in their ability to resist the attack of the pathogen. No disease symptoms were observed after one week from inoculation, while the susceptible seedlings non-treated with G 33 were severely infected. This proves that narcosis has no influence upon the effect of G 33, and that G 33 also has the capacity to act in narcotised host tissues.

The narcotised seedlings which were left in the open air recovered their healthy appearance after 2–3 days. Retreatment of these recovered seedlings with G 33 induced resistance against *Cladosporium cucumerinum*.

Repeated experiments gave similar results.

Photo 13 (plate IV) shows one of the experiments to determine the effect of narcotics and subsequent inoculation on seedlings treated with G 33. 1, 2, 3 and 4 represent: susceptible seedlings, susceptible seedlings treated with G 33, treated seedlings narcotised by ether, and treated seedlings narcotised by chloroform respectively.

The stage of infection in the figure is one week after inoculation.

Photo 14 (plate IV) illustrates the effect of inoculation with *Cladosporium cucumerinum* on seedlings which after recovery from narcosis by chloroform (bottle 3) or by ether (bottle 2) had been treated with G 33. Bottle 1 contains untreated susceptible seedlings.

It is clear, therefore, that while narcosis breaks down genetic resistance, it has no influence as such upon resistance induced by treatment with G 33.

This leads to the conclusion that both kinds of resistance are caused by different mechanisms.

The results of the effect of certain substances inhibitory to phosphorylation and respiration, and of narcotics, on genetic and induced resistance can be summarized as in the following table.

TABLE 4. The effect of certain substances inhibitory to phosphorylation and respiration, and of narcotics on the breakdown of genetic and G 33-induced resistance

Treatment with	Genetic resistance	G 33-induced resistance
The phosphorylation inhibitor		
2,4 dinitrophenol	only slight effect in "Mabro"	no effect
The respiration inhibitors		
sodium diethyl dithiocarbamate . .	no effect	no effect
sodium azide	no effect	no effect
Narcotics		
ether	breakdown	no effect
chloroform	breakdown	no effect

3. Infection by Cladosporium cucumerinum of narcotised genetically resistant seedlings treated with G 33

An attempt was made by the author to discover whether the treatment of the genetically resistant seedlings with G 33 would prevent breakdown of resistance in these seedlings by narcosis.

Hundred seedlings of "Mabro" were used in the experiment; 80 seedlings were treated with a solution of G 33 100 p.p.m. in the way described above with the susceptible seedlings. These seedlings were then narcotised by exposure to an atmosphere over a solution of ether 1.5% in water. The technique of narcotising the seedlings was as indicated above. Half of the narcotised seedlings were inoculated with an aqueous spore suspension of *Cladosporium cucumerinum*, while the other half was left uninoculated in the greenhouse for recovery. Susceptible seedlings of the variety "Lange gele tros" treated and untreated with G 33, together with the rest of "Mabro" seedlings (20 seedlings) were inoculated with the pathogen and set up as controls. All the inoculated seedlings were kept under optimum conditions for the progress of cucumber-scab.

After 7 days from inoculation the narcotised "Mabro" seedlings treated with G 33 remained resistant to the attack of the pathogen. They showed no symptoms of the disease. From all the seedlings included in the experiment only the susceptible seedlings untreated with G 33 showed normal symptoms of the disease.

The conclusion which can be drawn is that the genetic resistance of "Mabro" seedlings which doubtless was broken down as proved above was substituted by induced resistance through the action of G 33, hence the pathogen was impeded and no disease symptoms were revealed. The narcotised "Mabro" seedlings which were left uninoculated regained their natural appearance on exposure to open air.

C. DISCUSSION

It was proved that narcosis breaks down the genetic resistance in tissues of "Mabro" and "Proso". However, it fails to affect the induced resistance, in tissues of susceptible seedlings treated with G 33. The conclusion is that the mechanism of resistance in the genetically resistant varieties under investigation, is different from that in the susceptible variety made resistant by treatment with G 33.

It may also be concluded that the genetic resistance is dependent on the host metabolism. As the narcotics used are very unspecific and may have affected many metabolic processes, it is impossible to conclude from the present experiments which of these processes is responsible for the genetic resistance to *Cladosporium cucumerinum*. Probably, however, it is not respiration or phosphorylation.

Microscopical evidence shows that narcosis not only renders the genetically resistant tissues susceptible to the pathogen, but they have been altered to a point where over-susceptibility is reached.

The fact that G 33 is effective in the healthy as well as in the narcotised tissues is quite striking. This indicates that the effect of G 33 is also active in tissues the metabolism of which has been damaged.

Since induced resistance is not influenced by the inhibitors of synthetic processes and respiration applied in the present work, nor by narcotics such as ether and chloroform, the question still arises how the action of G 33 with the treated host tissues takes place. This question is not easy to answer and requires further and persistant investigations.

Any explanation of the induction of scab resistance by G 33, which supposes an action of this substance on the metabolism of the host is rendered improbable by the fact that this kind of resistance is not affected by narcosis. It seems more likely that the treatment of the plants with G 33 gives rise in the host tissues to some fungistatic or fungicidal substances, by which the progress of the fungus is inhibited. VAN RAALTE *et al.* (72) pointed out that it was very unlikely that G 33 itself could exert such a fungistatic action in the plant. If however, in the plant tissues the substance is slowly decomposed, a fungustatic substance might originate at a rate which is too low to cause much damage to the plant, but which is high enough to inhibit the hyphae of *Cladosporium*. That G 33 is broken down in the plant, is indicated by the fact revealed by VAN DER KERK *et al.* (69), that in tomatoes the epinasty caused by G 33 gradually disappears if the substance was only once applied. One is tempted to assume that the acetic acid side chain disappears, by which free dithiocarbamate, a strong fungicide would originate. A decomposition in this way seems chemically rather unlikely.

We may assume, however, that the G 33 molecule is easily translocated through plant tissues and that it gradually decomposes into two parts, one of which is a fungistatic or fungicidal substance.

The breaking down of resistance by narcosis in tissues of "Mabro" and "Proso" was only restricted to attack by *Cladosporium cucumerinum*. Other facultative parasites which are naturally not adapted to the cucumber plant, were not able to invade the narcotised tissues. This does not agree with the findings of BEHR (9), where narcotised resistant potato tubers were attacked by facultative parasites not-adapted to potato plants.

CHAPTER VI

THE EFFECT OF EXTRACTS FROM RESISTANT TISSUES ON THE GROWTH OF THE PATHOGEN

A. INTRODUCTION

The effect of chemical factors in determining internal resistance has been much investigated. An obvious line of research is to attempt to demonstrate, in the sap of resistant plants, the presence of substances inhibitory or toxic to fungi. SCHMIDT (60) found that species of *Solanum* resistant to *Cladosporium fulvum* contain in their sap a principle antagonistic to the fungus. He gave for this principle of unknown composition the name "prohibitin". REYNOLDS (58) discovered a glucoside which produces hydrocyanic acid upon hydrolysis, in strains of flax resistant to *Fusarium lini*. In many extracts it completely inhibits the pathogen at the normal concentrations. LINK and WALKER (46) report that catechol, along with protocatechuic acid, present in the host sap, appear to be the chief toxic substances that enable the pigmented onion to resist the invasion of the fungus *Colletotrichum circinans*, the organism responsible for "smudge" disease of onions. RAMSEY *et al.* (57) claim that a chemical (probably protocatechuic acid) associated with the pigments in water extracts of the dry outer scales of coloured varieties of onions proved toxic to spores of *Diplodia natalensis*.

CAMPANILE (21) worked on conditions favourable to attack and development of *Helminthosporium allii* on garlic. He concluded that while the red pigment undoubtedly plays a part in checking the fungus, it is not the sole factor responsible for the resistance of the coloured variety.

In the present chapter an attempt is made to discover whether the extracts from resistant tissues, are inhibitory to the growth of *Cladosporium cucumerinum*. The tissues involved, were those of "Mabro" and "Proso", the two scab-resistant varieties under investigation, and those of the old vegetative parts of the susceptible variety "Lange gele tros".

B. EXPERIMENTAL RESULTS

Older plants of the two resistant varieties "Mabro" and "Proso", and of the susceptible variety "Lange gele tros", when possessing 2–3 full-grown leaves, and two young and immature ones, were inoculated by spraying the whole plant with an aqueous spore suspension of *Cladosporium cucumerinum*. The inoculated plants were kept under favourable conditions for infection; i.e. a rather low temperature (about 20 °C), and high humidity in the way described in previous chapters.

Seven days after inoculation the tips of the susceptible plants were cut off and discarded, for they were severely infected. The rest of the plants which was highly resistant, was used for the purpose of the present investigation.

In case of the two resistant varieties the whole plant was utilised.

The sap from the resistant tissues was extracted as follows: the plants were subjected to pressure under a hydraulic press. The juice was collected and filtered through filter paper, or centrifuged at the speed of 12,000 R.P.M. for 20 minutes, to remove the solid particles. The resulting filtrate was then filtered through a sterilized Seitz filter to get rid of any contaminating micro-organisms. The eventual sterile filtrate was poured off in a sterile bottle and kept for use.

Each of the three extracts; i.e. the extract from "Mabro" tissues, the extract from "Proso" tissues, and the extract from older tissues of "Lange gele tros", was mixed with a hot doubly concentrated sterilized malt agar in equal proportions (10 gr. malt Difco per litre, 56 gr. agar agar per litre, were the concentrations used in preparing the malt agar).

Five 20cc. sterilized test tubes, each containing about 4cc. from ("Mabro" extract + malt agar), were laid down almost horizontally to obtain a wide surface of the medium. The same was repeated with the other two mixtures. As controls 5 test tubes were set up in the same way, with the difference that the medium was composed of a mixture of malt agar and distilled water in 1 : 1 proportion. The tubes were left until the medium was properly solidified, then the latter was inoculated with *Cladosporium* spores and pieces of mycelium, and the tubes were kept in an incubator at 23 °C.

After 4 days the growth of the fungus on the different media was observed. In no case, was there an inhibitory effect of the extract on the growth of the fungus. The latter grew at the same rate and density on the surface of all the tested media, whether the malt agar was mixed with any of the 3 extracts or not.

C. DISCUSSION

The normal growth of *Cladosporium cucumerinum* on the medium containing extracts from tissues of "Mabro" and "Proso", proved that these extracts do not contain an inhibitory or toxic principle or substance to the growth of the fungus. This result was also obtained with the extract from tissues of the older parts of the susceptible variety "Lange gele tros".

In chapter V it was concluded that in "Mabro" and "Proso" the genetic resistance is dependent on the host metabolism. It has been suggested by the author that this resistance is due to a principle present in the host tissues that inhibits the growth of the pathogen. The non-inhibitory effect of the extracts from these varieties indicates that this principle if it exists, is temporarily active, and that it retains for a short time its inhibiting power, within the host. Therefore, the permanent resistance shown by "Mabro" and "Proso" tissues may depend on the continuity of production of the assumed principle, rather than the maintenance of its inhibitory effect once it is produced.

GOTHOSKAR *et al.* (32) discovered a similar case with tomato tissues resistant to *Fusarium lycopersici*. They claimed that a substance is continuously formed in the host tissues, which is toxic to the fungus. They added that this substance must be either very labile or continuously metabolized to nontoxic materials, since extracts of resistant plants are not toxic to the fungus.

It is quite possible that a principle having the same characters also exists in tissues of the older parts of the susceptible variety "Lange gele tros".

SUMMARY OF PART II

1. An attempt was made to discover whether the suggested principle inhibitory to growth of *Cladosporium cucumerinum* within cucumber tissues, is produced in both genetic and induced resistance by similar or by different mechanisms.

2. The phosphorylation inhibitor 2,4 dinitrophenol, and the two respiratory inhibitors sodium diethyldithiocarbamate and sodium azide have no influence on genetic or on G 33 induced resistance.

3. The genetically resistant seedlings if exposed to an atmosphere over a solution of ether 1.5% or of chloroform 0.25% in water, lose their resistance to *Cladosporium cucumerinum*. They become severely infected 2 days after inoculation, and abnormal superficial and internal mycelial growth occurs.

4. The narcotised seedlings of "Mabro" and "Proso" if inoculated with *Penicillium italicum* or *Botrytis cinerea* remain resistant to these fungi. This indicates that the mechanism of genetic resistance of cucumber plants to *Cladosporium cucumerinum* is different from that of natural resistance of the same plants to *Penicillium italicum* or *Botrytis cinerea*.

5. Narcosis has no influence on the effect of G 33. The treated seedlings narcotised by ether 1.5% or chloroform 0.25% retain their induced resistance. In genetically resistant seedlings resistance of which has been broken down by narcosis, chemical resistance could be induced by treatment with G 33. This implies that genetic and induced resistance are caused by different mechanisms.

By applying the above narcotics, genetic resistance was proved to be related to host metabolism, while induced resistance was not.

6. Neither the expressed sap from resistant tissues of "Mabro" and "Proso" nor that from the older parts of the susceptible variety "Lange gele tros" proved to contain an inhibiting principle to the growth of the pathogen.

SAMENVATTING

DEEL I

Microscopisch onderzoek van gastheer en parasiet in het weefsel van voor Cladosporium vatbare, met G 33 resistent gemaakte, en genetisch resistente komkommerplanten

1. Een anatomisch onderzoek werd verricht naar de reactie op infectie door *Cladosporium cucumerinum* van komkommerplanten van een vatbaar ras, – niet behandeld ofwel met G 33 resistent gemaakt –, en van 2 genetisch resistente rassen: "Mabro" en "Proso".

In het volgende wordt verstaan onder: "vatbare planten" of "niet behandelde planten" de planten van het vatbare ras, die niet met G 33 zijn behandeld; "behandelde planten" de planten van het vatbare ras, die met G 33 zijn behandeld en resistent gemaakt; "resistente planten" de planten van de genetisch resistente rassen.

G 33 is S-carboxymethyl-N, N-dimethyldithiocarbaminaat:

$$(CH_3)_2\,N\text{–}C(=S)\text{–}S\text{–}CH_2COOH.$$

2. De sporen van de schimmel kiemen en vormen goed ontwikkelde appressoriën op de intacte epidermis (fig. 1) of in wonden in de cotylen, zowel van vatbare planten, als van behandelde of van genetisch resistente planten.

3. Bij de vatbare planten doorboorde de schimmel binnen 48 uur de epidermis (fig. 2). Binnendringen door de stomata (fig. 3) geschiedde veel minder vaak. In het weefsel gedraagt de schimmel zich als volgt:

a. De hyphen groeien intercellulair tussen de palissadecellen door tot ze het sponsparenchym bereiken. Hier vertakken zij zich en verspreiden zich door de intercellulairen. Pas als de cellen beginnen af te sterven dringen de hyphen er in binnen. Dan groeit de schimmel dus ook intracellulair.

b. In het intercellulaire stadium liggen de hyphen meestal dicht tegen de celwanden aan (fig. 4); daar waar een hyphe loodrecht op een celwand stuit zwelt zijn top op (fig. 5).

c. Enige dagen na het begin van de infectie worden in het lumen van een deel van de palissadecellen sterk lichtbrekende structuren zichtbaar (plaat I, foto 1). Deze hebben de vorm van onregelmatige cirkels of ellipsen. Waar deze structuren aanwezig waren, bleek een gedeelte van de tussenwand tussen twee aangrenzende cellen overlangs gespleten te zijn, terwijl hier de cellen van elkaar losgeraakt waren. De lichtbrekende ring bevond zich op de grens van dat deel waar de cellen nog door een gemeenschappelijke wand waren verbonden en de plaats waar de twee helften van de oorspronkelijke celwand uiteengeweken waren (fig. 6 en 7). Vermoedelijk vormt de schimmel een pectine-oplossend enzym, dat de middenlamel ten dele oplost.

d. De hyphen in de intercellulairen veroorzaken geen aantoonbare veranderíng van de cellulose van de celwand, ook niet daar waar ze dicht tegen de celwand aanliggen.

e. Het enige aanwijsbare effect van de schimmel op de celwand was op de pectine. Als dunne schijfjes aangetast weefsel gedurende 20 minuten met warm 2% zoutzuur werden behandeld, kleurden de celwanden van de delen waar zich hyphen van de schimmel in bevonden nog duidelijk rood met ruthenium-rood. In gezonde weefsels of in weefsels van een zieke plant, waarin geen hyphen aanwezig waren, hadden de celwanden na een dergelijke behandeling met zoutzuur het vermogen om met rutheniumrood te kleuren verloren. Vermoedelijk scheidt de schimmel een stof af, die de pectine physisch of chemisch zo verandert, dat hij oplosbaar wordt in zuur.

f. In de intercellulairen of in de cellen van zieke weefsels komt in kleine hoeveelheden "gom" voor als een homogene gele stof. Deze substantie kleurt niet met ruthenium rood of met chloorzinkjodium.

g. De hyphen leggen de afstand tussen de geïnoculeerde boven- en de beneden epidermis, al naar de uitwendige omstandigheden, in 3–6 dagen af.

h. In het laatste stadium van de ziekte, als de cellen van de gastheer necrotisch zijn en beginnen te verschrompelen, komen de conidiophoren in massa door de resten van de bovenepidermis heen naar buiten. Op de beneden epidermis verschijnen er veel minder, gedeeltelijk uit de stomata, gedeeltelijk na doorboren van de cuticula (fig. 8).

Als in kunstmatige wonden werd geïnoculeerd, verliep de infectie niet anders dan hierboven werd beschreven.

4. Door de intacte epidermis van de cotylen van met G 33 behandelde kiemplantjes drong de schimmel zelden naar binnen. In een enkel geval werd binnendringen door een huidmondje waargenomen, maar de hyphe was hier blijkbaar

niet verder gekomen dan de inwendige ademholte onder het huidmondje (fig. 9). Als de oppervlakte door krassen werd beschadigd en de gemaakte wonden werden geïnoculeerd, groeiden uit de appressoria hyphen door de dode cellen van de wond heen, doch deze hyphen kwamen niet verder dan 1–2 cellagen diep in het gezonde weefsel (fig. 10). De aanwezigheid van een kleine hoeveelheid mycelium in de wond, veroorzaakte het bovengenoemde effect op de celwanden rond de beschadigde plek.

5. Bij alle oudere, vatbare planten werd de top, bestaande uit de nog niet volwassen bladeren, hun bladstelen, en de jonge internodiën hevig aangetast, terwijl de rest van de plant resistent was onder de omstandigheden van de proef.

6. Bij intacte of beschadigde niet volwassen bladeren komt infectie op soortgelijke wijze tot stand als bij de cotylen. De enige verschillen bestaan uit de hoeveelheid van de door het protoplasma van de waardplant uitgescheiden stoffen en de reacties op bepaalde kleurstoffen, die chemische verandering van de cellulose van de celwanden te weeg brengen, uit de mate van beschadiging van de onderste epidermis, en uit die van de invloed van de schimmel op de pectine van de celwand.

7. Op het intacte oppervlak van de jonge internodiën (of bladstelen) vindt normale sporenkieming en vorming van appressoriën plaats. Binnen 48–72 uur na de inoculatie wordt de cuticula doorboord. Aanvankelijk groeit ook hier de schimmel eerst intercellulair, doch eerder dan bij de cotylen schijnt hij tot een intracellulaire groeiwijze over te gaan (fig. 16). De top van hyphen, die de wand van een intacte cel ontmoeten, zwelt op. Na het tot stand komen van de intracellulaire groei ontstaan soms lysigene holtes in het parenchym van de centrale cylinder. Deze zijn gevuld met een homogene gele stof (plaat I, foto 2).

8. Door de schrijver werd de naam van extra-fasciculair "phloeem" gegeven aan strengen van lange dunwandige cellen, die dicht met protoplasma gevuld waren (plaat I, foto 3). Het was bekend, dat deze strengen bij de Cucurbitaceeen voorkomen. Ze worden door sommige schrijvers als zeefvaten beschouwd. Na infectie waren deze cellen gevuld met een homogene gele substantie (plaat I, foto 5) terwijl de normale zeefvaten hiervan vrij bleven. Dit wijst op een physiologisch verschil tussen deze weefsels. Microchemische reacties toonden aan, dat de 3 stoffen die in de zieke stengel (of bladsteel) worden gevonden, t.w. de granulaire substantie in de cellen, de homogene stof van de lysigene holtes en de homogene stof in het extra-fasciculaire "phloeem" chemisch verschillend waren.

9. In de vaten werden alleen hyphen gevonden indien ze door hevig aangetaste cellen waren begrensd. Infectie vanuit een wond gaf dezelfde verschijnselen als de hierboven beschrevene. Oude internodiën (of bladstelen) waren zeer resistent tegen binnendringen van de schimmel, ook als deze in wonden werd aangebracht. De dikte van de cuticula speelt hierbij geen rol; aangetoond kon worden, dat de resistentie zowel in de epidermis als in de dieper gelegen weefsels zetelt.

10. Een behandeling van komkommerplanten met G 33 maakt de jonge delen grotendeels resistent tegen *Cladosporium* aantasting. Na de vorming van appressoriën drongen geen hyphen door de intacte epidermis van met G 33 behandelde planten heen. Slechts in enkele gevallen geschiedde dit wel, doch de groei van de schimmel was dan langzaam en de aantasting was van beperkte omvang. Onder de aantasting hadden de cellen gelegenheid gehad een kurkweefsel te vormen (plaat I, foto 6 en plaat II, foto 7).

In geïnoculeerde wonden, die door krassen waren gemaakt, bleven de hyphen beperkt tot de dode cellen langs de wond.

11. Uit de resultaten verkregen met de behandeling van kiemplantjes of van oudere planten met G 33 volgt, dat:

a. de door G 33 geinduceerde resistentie zowel in de epidermis als in dieper gelegen weefsels zetelt,

b. G 33 alleen effect heeft in cellen, welke onder het microscoop geen afwijkingen vertonen,

c. de invloed van G 33 niet tot buiten de cuticula reikt.

12. De beide genetisch resistente rassen "Mabro" en "Proso" bleken bij microscopisch onderzoek zeer resistent, zowel in het kiemplant-stadium als later. Op het intacte oppervlak van cotylen, bladeren, bladstelen en stengels kiemden de sporen en vormden normale appressoriën. De laatsten vormden echter meestal geen infectie-hyphen. In de enkele gevallen waarin hyphen in het weefsel van de gastheer werden waargenomen konden zij veelal niet verder groeien dan de epidermis (fig. 18), of kwamen, bij een zeer geslaagde invasie, niet verder dan voorbij de eerste laag palissadecellen (fig. 11 en 17). De cellen van de gastheer in de buurt van deze hyphen kunnen wel een verandering vertonen, doch volledig afsterven of verschrompelen van deze cellen kwam niet voor. In stengels en bladstelen vindt men altijd een kurkweefsel rond de kleine geïnfecteerde plek. Een dergelijk weefsel ontstaat rond iedere wond.

Als krassen, gemaakt in de epidermis van een willekeurig deel behalve in die van de cotylen, werden geïnoculeerd, groeiden de hyphen uit de appressoriën slechts in de dode cellen, en slechts zeer zelden tussen de aangrenzende gezonde. Bij de cotylen kunnen in dat geval de hyphen wel het gezonde weefsel binnendringen, doch hun groei wordt vertraagd of gestuit tengevolge van een mechanisme, dat waarschijnlijk bij de twee varieteiten verschillend is (fig. 13 en 15). De chemische reacties van de celwanden in de resistente rassen verschillen bij de intacte plant niet van die in de niet resistente.

Het aanbrengen van een wond door krassen van de epidermis van een cotyl of van het blad heeft bij resistente rassen echter tot gevolg, dat de wanden van de gezonde cellen rond de wond na behandeling met 2% HCl nog met ruthenium-rood kleuren. De aanwezigheid van de schimmel is hiervoor niet noodzakelijk.

13. In de genetisch resistente planten, evenals bij de met G 33 behandelde, wordt de groei van de schimmel door een onbekend mechanisme geremd. Deze remming is niet aanwezig op het buiten-oppervlak van de planten.

DEEL II

Onderzoekingen over de aard van de genetische en de chemisch-geïnduceerde resistentie tegen Cladosporium cucumerinum

1. Onderzocht werd, of de groeiremming van *Cladosporium cucumerinum* in het geval van genetisch veroorzaakte en in dat van geïnduceerde resistentie door hetzelfde mechanisme werd veroorzaakt.

2. 2,4-dinitrophenol en de ademhalingsvergiften natrium diaethyldithiocarbaminaat en natrium azide hebben geen invloed op de genetische en de door G 33 geïnduceerde resistentie.

3. Genetisch resistente kiemplantjes verliezen hun resistentie tegen *Cladosporium cucumerinum* als ze worden blootgesteld aan een atmospheer boven 1.5% ether of boven 0.25% chloroform in water. Ze worden dan twee dagen na inoculatie hevig aangetast, waarbij een zeer abnormaal infectiebeeld ontstaat (plaat II, foto 8 en 9).

4. Genarcotiseerde kiemplantjes van "Mabro" en "Proso" die met *Penicillium italicum* of *Botrytis cinerea* worden geïnoculeerd blijven resistent tegen deze schimmels. De natuurlijke weerstand van de planten tegen deze schimmels berust dus op een ander mechanisme, dan die tegen *Cladosporium cucumerinum*.

5. Narcose heeft geen invloed op het effect van G 33. Met ether of chloroformdamp genarcotiseerde kiemplantjes blijven resistent, als ze met G 33 behandeld zijn. Wanneer de resistentie van genetisch resistente planten door narcose werd vernietigd, kon een nieuwe, chemische resistentie van de planten te voorschijn geroepen worden door behandeling met G 33. De genetische resistentie is dus afhankelijk van de stofwisseling van de gastheer, terwijl de chemisch-geïnduceerde weerstand dit niet schijnt te zijn.

6. Noch in perssap van de resistente "Mabro" en "Proso", noch in dat van de vatbare "Lange Gele Tros" werd een stof gevonden, die de groei van de schimmel in vitro remde.

ACKNOWLEDGEMENTS

This work was carried out under the direction of Prof. Dr. A. J. P. OORT, to whom I am deeply indebted for his guidance and critical advice.

The suggestions and the continued interest of Dr. M. H. VAN RAALTE are greatly appreciated.

I should like also to express my thanks to Dr. Ir. A. P. KOLE, Mr. F. SCHEYGROND and Mr. B. J. VAN TONGEREN for their help in preparing the photographs and the figures.

The author acknowledges the assistance of the T.N.O. Research Team for Internal Therapy of Plant Diseases in providing the G 33.

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Plate I

Photo 1. Cross section of a susceptible cotyledon, showing a highly refractive ellipse in the lumen of a palisade cell, a structure usually present in cells of infected palisade tissue. Phase contrast. ×250.

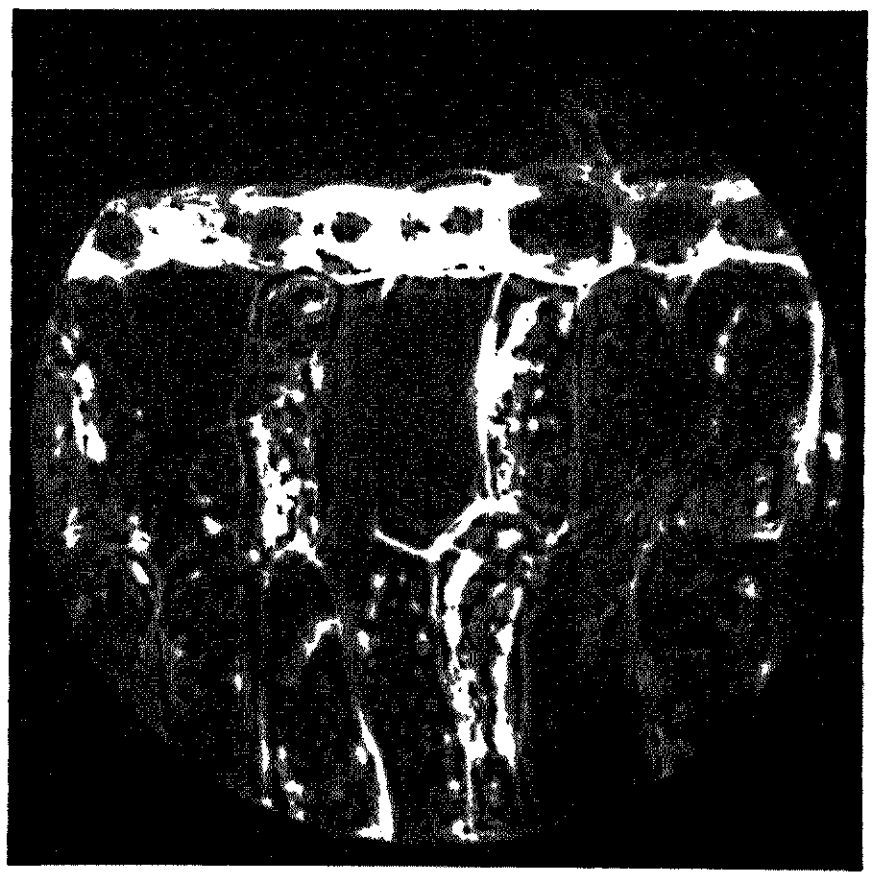
Photo 2. A lysigenous cavity formed out of several parenchyma cells in the central cylinder of an infected susceptible stem at an advanced stage of the disease. Note the presence of hyphae inside the cavity. ×67.

Photo 3. A part from a cross section in an old susceptible stem showing the extra-fascicular "phloem" strands running mainly in longitudinal direction, hence they appear as angular cells deeply stained, for they are filled with a non-granular substance. These strands are connected with other ones that run more or less horizontally. Of these one is visible in the figure, the elements of which are free of the deeply coloured substance. ×42.

Photo 4. A part from a longitudinal section in a healthy young susceptible stem demonstrating the elements of the extra-fascicular "phloem" quite free from the non-granular substance which fills the same elements in diseased young stems (photo 5). Note the short connecting horizontal strand between two longitudinal ones. ×68.

Photo 5. The extra-fascicular "phloem" elements as they appear longitudinally in a diseased young susceptible stem. The elements become filled with a substance that reacts as a mixture of different chemical compounds. ×66.

Photo 6. One of the few cases in which penetration into a young petiole from a susceptible plant treated with G 33 did occur. After 7 days from inoculation the infection is restricted only to a few layers of the cortex. The host cells have not completely collapsed, and the hyphae, which are merely intercellular are rarely observed. The whole small lesion is surrounded by actively dividing cells which form a wound tissue. ×68.



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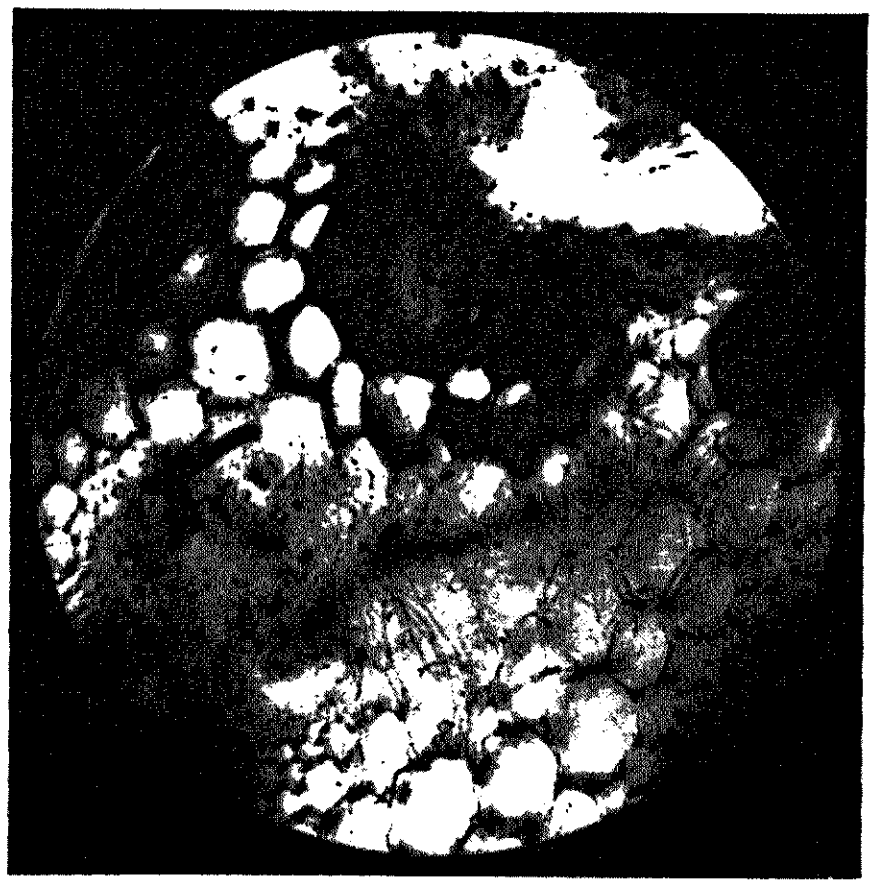

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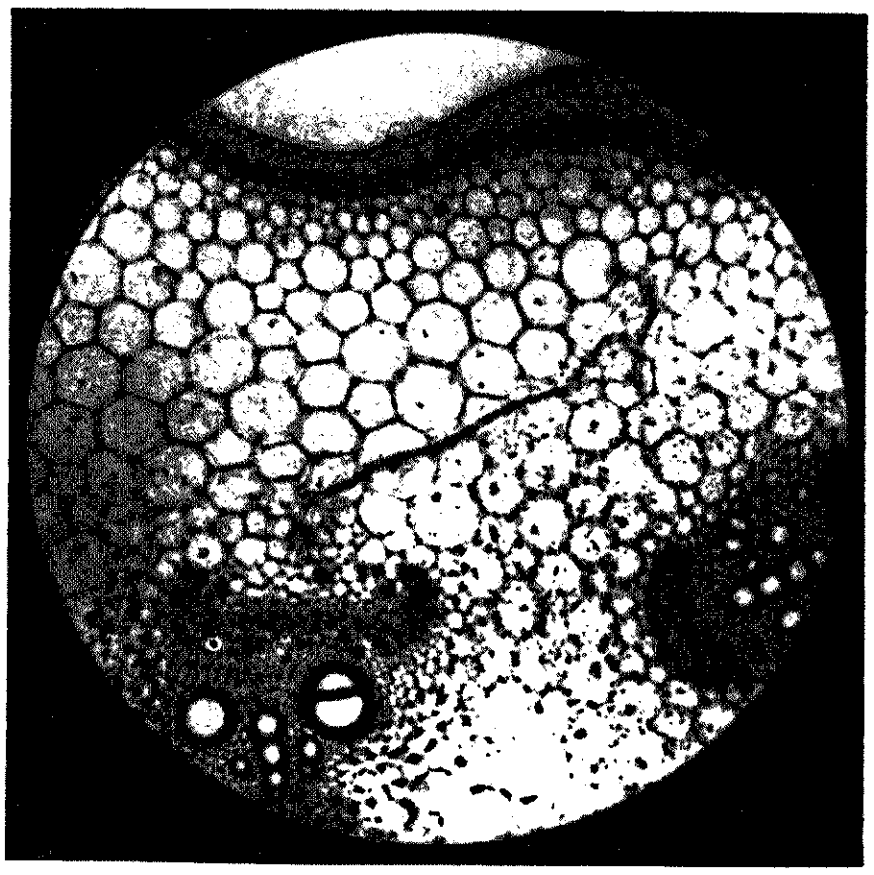

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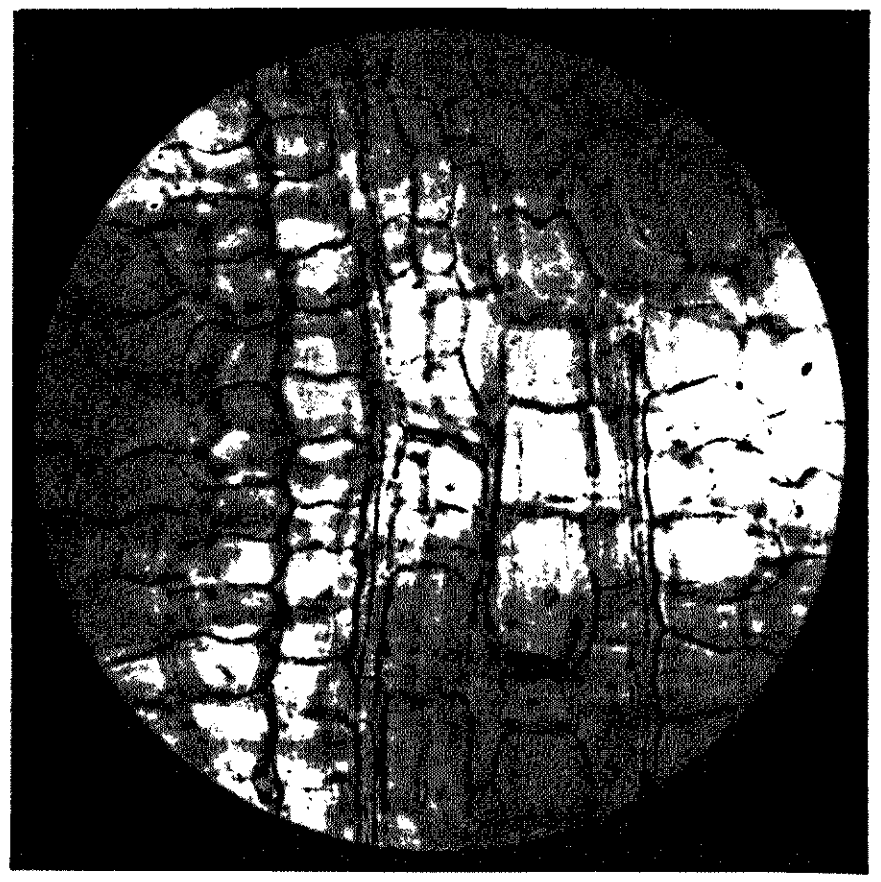

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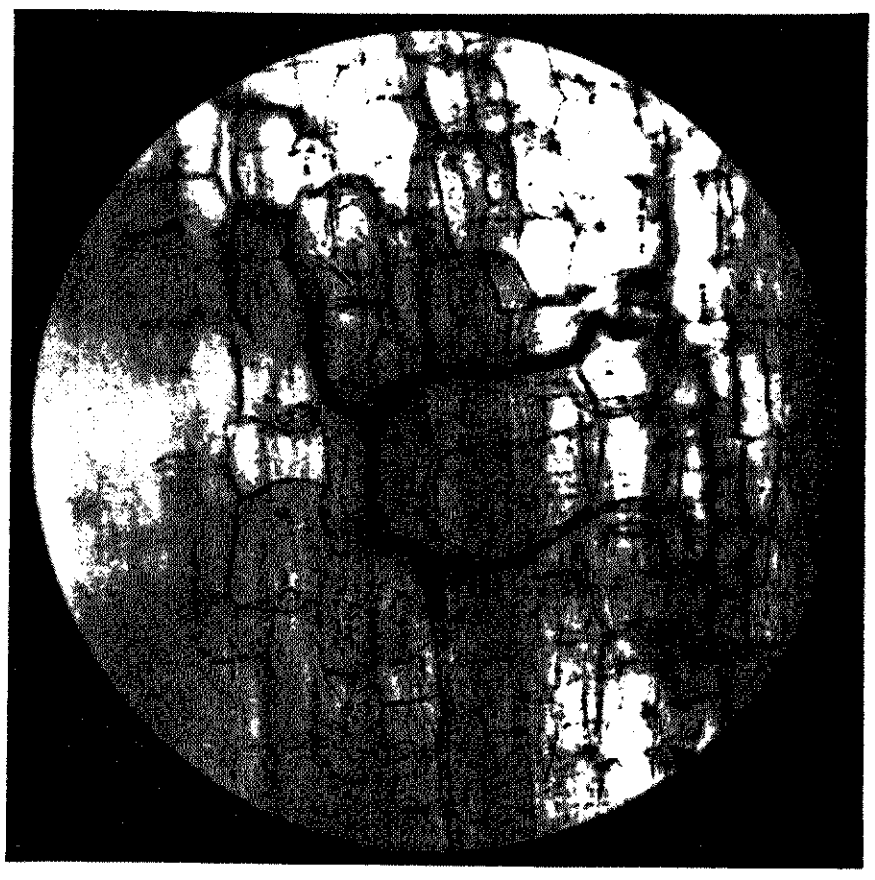

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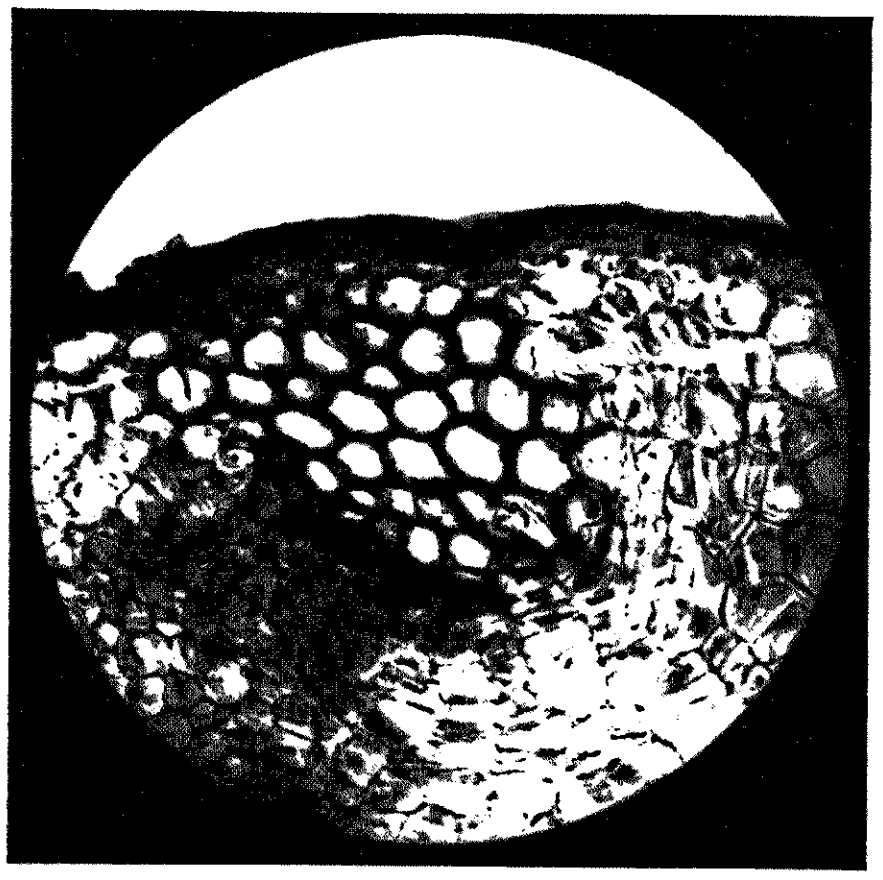

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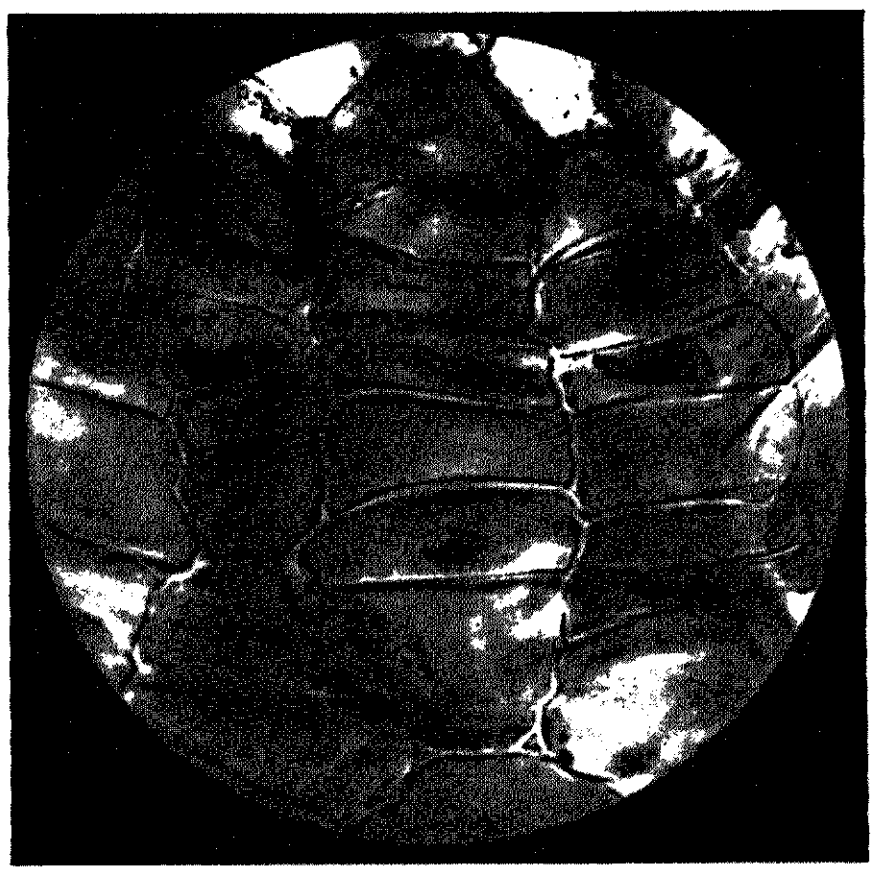

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PHOTO 7. The wound tissue in photo 6 is demonstrated at a higher magnification. ×270.

PHOTO 8. A cross section of a narcotised cotyledon of "Mabro" fixed 2 days after inoculation. The height of the abnormal superficial growth of the mycelium is nearly equal to the cross section of the cotyledon itself. ×19.

PHOTO 9. The superficial mycelial growth as it appears at a higher magnification. It is associated with an abundant growth of the hyphae inside the host tissues. Two days after inoculation the host cells were greatly concealed by this extraordinary internal growth of the pathogen. ×70.

8

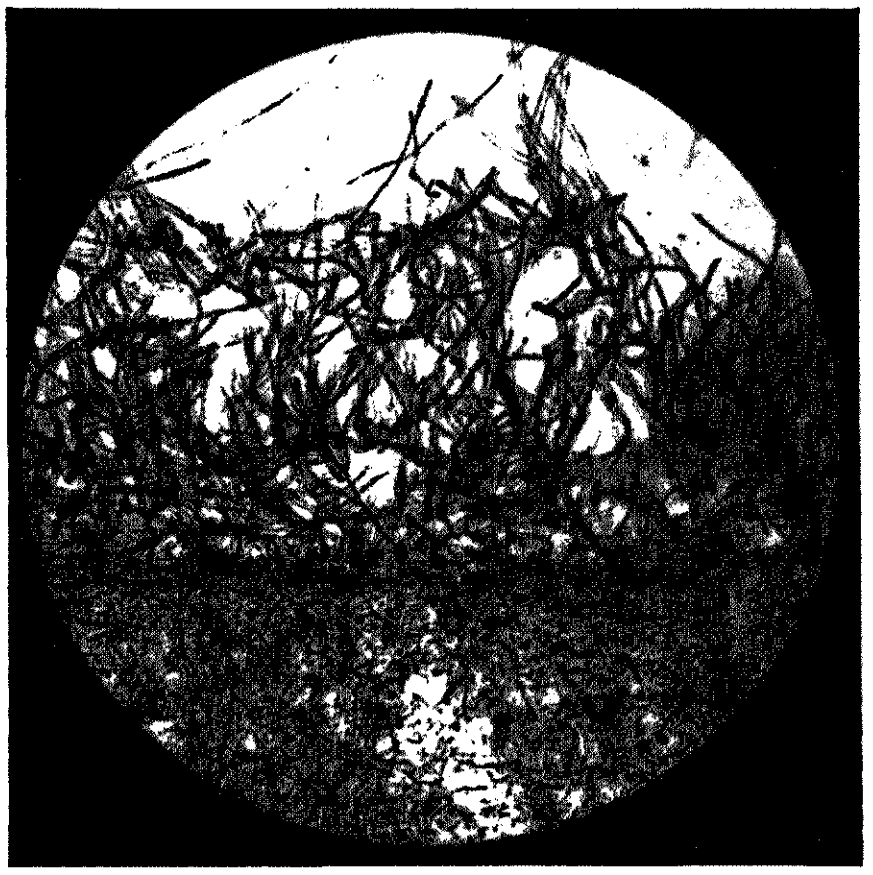

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PHOTO 10. One of the experiments where susceptible seedlings treated with G 33 were transferred to solutions of Na DDC and Na AZ, then inoculated and observed after a week for the development of the disease.

1. Inoculated susceptible seedlings.
2. Susceptible seedlings treated for 2 days with G 33, then inoculated.
3. G 33-treated seedlings transferred to Na AZ 10^{-4}M solution, then inoculated.
4. G 33-treated seedlings transferred to Na AZ 10^{-5}M solution, then inoculated.
5. G 33-treated seedlings transferred to Na DDC 10^{-5}M solution, then inoculated.

In 1. severe infection takes place.
In 2, 3, 4, 5 no disease symptoms are observed.

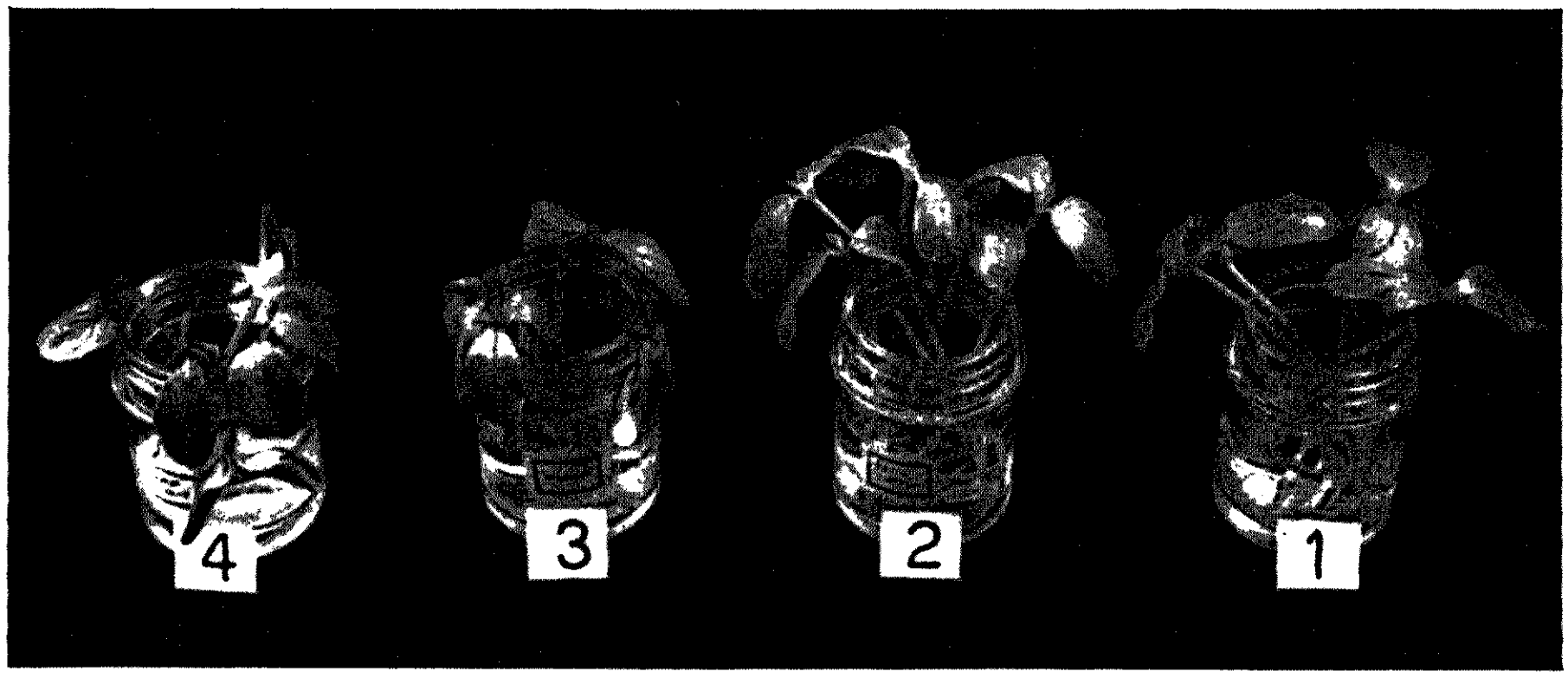

PHOTO 11. One of the experiments where susceptible seedlings were treated with a mixture of (G 33 + DNP) for 2 days, then inoculated, and the result observed after a week.
1. Susceptible seedlings treated with G 33 for 2 days, then inoculated.
2. Susceptible seedlings treated with a mixture of (200 p.p.m. G 33 + 2×10^{-5}molar DNP) for 2 days, then inoculated.
3. Inoculated susceptible seedlings.
4. Susceptible seedlings treated with DNP 10^{-5} molar for 2 days, then: noculated.

In 1, 2 no disease symptoms are observed.
In 3, 4 severe attack of the seedlings by the disease.
Similar results were obtained when applying mixtures of (G 33 + Na AZ) or (G 33 + Na DDC).

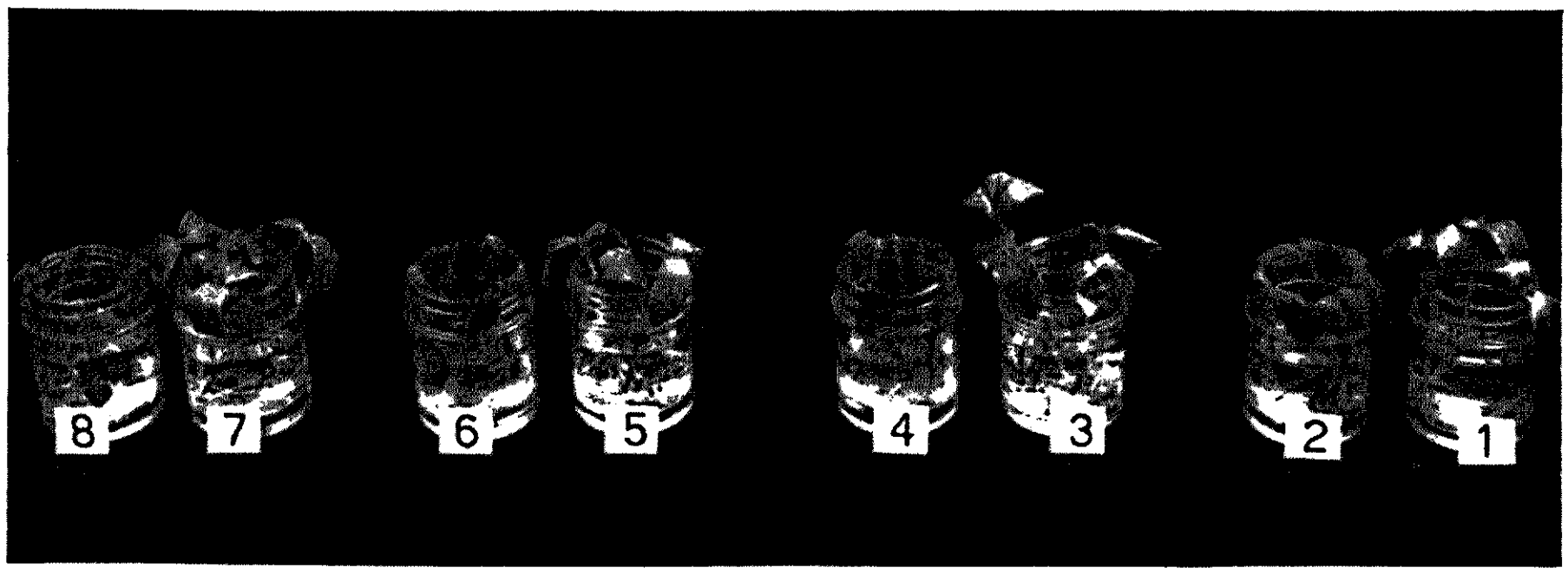

PHOTO 12. One of the experiments to determine the effect of narcosis on genetic resistance.
1. Complete recovery of narcotised "Mabro" seedlings after the lapse of 48 hr. on exposure to the open air. The narcotic applied is chloroform 0.25 %.
2. Severe infection 2 days after inoculating "Mabro" seedlings narcotised with 0.25% chloroform.

3, 4. The same as in 1, 2 with the only difference that "Proso" seedlings are used instead of "Mabro" seedlings.

5. Complete recovery of "Mabro" seedlings narcotised with 1.5% ether on exposure for two days to the open air.
6. Severe infection 2 days after inoculating narcotised "Mabro" seedlings with 1.5% ether.

7, 8. The same as in 5, 6 except that "Proso" seedlings were utilized instead of "Mabro" seedlings.

Plate IV

Photo 13. One of the experiments to determine the effect of narcosis on susceptible seedlings treated with G 33. The stage of infection is one week after inoculation.

1. Susceptible seedlings severely attacked.
2. Susceptible seedlings treated with G 33 for 2 days then inoculated. No disease symptoms were developed.
3. Susceptible seedlings treated with G 33 for 2 days, then exposed to an atmosphere of 1.5% ether for 14 hr. while still in the G 33 solution. The seedlings were then transferred to distilled water and inoculated. No symptoms of the disease were observed.
4. The same as in 3 except that chloroform 0.25% was applied instead of ether. Also no disease symptoms were developed.

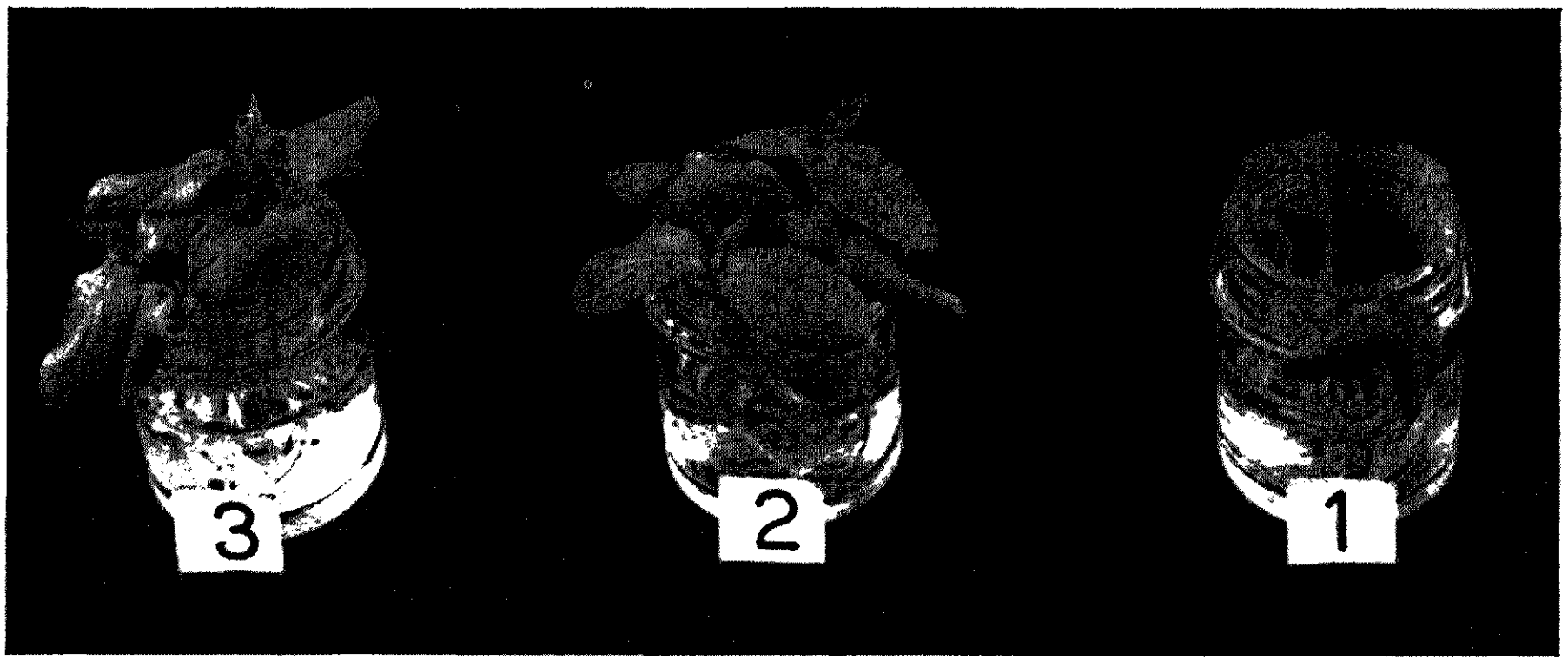

Photo 14. Illustrates the result one week after inoculating recovered and then G 33-treated susceptible seedlings prenarcotised by chloroform (bottle 2) and ether (bottle 3). Bottle 1 contains normal susceptible seedlings as control. The seedlings in 1 are greatly damaged, those in 2 and 3 are not diseased.