A. van Dijkman

Natural resistance of tomato plants to Cladosporium fulvum
A biochemical study

Natural resistance of tomato plants to Cladosporium fulvum *A biochemical study*

Typografie Kees Nieuwenhuijzen GVN

Stellingen

I De resistentie op basis van een hypersensitieve reactie van planten tegen fytopathogene schimmels berust vermoedelijk op een primaire reactie van excretie producten van de schimmels en de celmembraan van de plant.

Dit proefschrift. Hoofdstuk IV en VII.

II De rol die aan tomatine wordt toegeschreven in sommige plant-pathogeen combinaties is tot nu toe niet bewezen.

Kern, H., Phytopathol. Z., 19 (1952) 351.
Langcake, P., et al., Physiol. Plant Pathol., 2 (1972) 17.
Dit proefschrift. Hoofdstuk V.

III De mening van Macko en medewerkers dat polyacrylamide disc gel electrophorese gebruikt kan worden om fysiologische rassen van *Puccinia graminis* var. *tritici* en van andere fytopathogene schimmels te onderscheiden, is op grond van zijn experimenten voorbarig.

Macko, V., et al., Phytopathol. Z., 58 (1967) 122.

IV Het is onduidelijk waarom sommige fytopathologen de 'resistentie' van planten tegen saprofyten en niet-pathogenen trachten te verklaren.

Kuc, J., Ann. Rev. Phytopathol., 20 (1966) 337.
Allen, E. H. en Kuc, J., Phytopathology, 58 (1968) 776.

V Resistentie inductie van *Candida albicans* tegen pimaricine en nystatine is gekoppeld aan verlies van pathogeniteit.

Winner, H. I. en Athar, M. A., 10th Intern. Congr. Microbiol., 1970, Mexico, p. 149.
Petru, M. en Oostendorp, J. G., in voorbereiding.

VI Het veelvoud van copieen van delen van het DNA in het genoom zou een functie kunnen hebben als genetische reserve tegen spontane mutaties. Geleidelijke accumulatie van deze mutaties hebben veroudering tot gevolg.

Medvedev, Zh. A., Nature, 237 (1972) 453.
Holliday, R. en Tarrant G. M., Nature, 238 (1972) 26.
New Scientist van 29-6-72 en 13-7-72. Resp. p. 726 en 70.

VII De activiteit van indolazijnzuur oxydase, gemeten in een weefselhomogenaat, is geen bruikbare maat voor het vermogen van dit weefsel om endogeen indolazijnzuur af te breken.

Raa, J., Physiol. Plantarum, 25 (1971) 130.
Galston, A. W. en Davis, R. J., Science, 163 (1969) 1288.

VIII Volledig gedifferentieerde hormoon producerende cellen van de hypophyse voorkwab van de muis, kunnen overschakelen op de productie van een ander hormoon wanneer daaraan in het lichaam behoefte bestaat.

Dingemans, K. P., J. Ultrastructure Res., 26 (1969) 480.
Dingemans, K. P., Congr. Intern. Electr. Microscopy, VII, 1970, Grenoble, p. 563.

IX Het bestrijden van schimmelgroei op voedsel is niet alleen gewenst uit esthetisch oogpunt maar veel meer een noodzaak uit gezondheids-overwegingen in verband met het gevaar van mycotoxinen.

Van Walbeek, W., et al., Can. J. Microbiol., 14 (1968) 131.
Shank, R. C., et al., Food Cosmet. Toxicol., 9 (1971) 501.
Wogan, G. N., Federation Proc., 27 (1968) 93.

X Het behandelen van vergiftigingen door zware metalen met chelaten zonder voorafgaand onderzoek naar een eventueel gevaarlijke herverdeling in het lichaam, is onjuist.

Kamerbeek, H. H., Acta Med. Scan., 189 (1971) 149.

XI In een toom hennen is de sociale rangorde bepalend voor de mate van agressiviteit en de grootte van de ei-productie.

van Dijkman-Nieuwenhuijzen, E. H., et al., Bijdrage tot de Dierkunde, 37 (1968) 81.

XII Om in de toekomst de tot nu toe periodiek optredende boter en kaas overschotten te vermijden, is een structurele verandering van het productie assortiment van de zuivelindustrie noodzakelijk. Een doelgerichte marketing kan deze verandering mede bewerkstelligen.

Frietema, H. J., Econ. Stat. Ber., 57 (1972) 645.

XIII Bij de bestrijding van de stedelijke milieuvervuiling wordt de hond *(Canis familiaris)* bewust vergeten.

XIV Hallucinaties door het gebruik van grote hoeveelheden wijn vormen een gerede verklaring voor de ommezwaai van Picasso van de zogenaamde 'blauwe' periode naar de deformatieve.

A. van Dijkman
25 september 1972

Natural resistance of tomato plants to Cladosporium fulvum
A biochemical study

Proefschrift

ter verkrijging van de graad van doctor in de Wiskunde en Natuurwetenschappen aan de Rijksuniversiteit te Utrecht, op gezag van de Rector Magnificus Prof. Dr. Sj. Groenman, volgens besluit van het College van Dekanen in het openbaar te verdedigen op maandag 25 september 1972 des namiddags te 4 uur precies door

Antoon van Dijkman

geboren op 18 januari 1940 te Amsterdam.

Drukkerij Gebr. Janssen n.v. Nijmegen 1972

Promotoren:

Prof. Dr. K. Verhoeff
Prof. Dr. G. J. M. van der Kerk

Dit proefschrift werd bewerkt onder leiding van
Dr. Antje Kaars Sijpesteijn, Hoofd van de
Biochemische en Microbiologische Afdeling van het
Organisch Chemisch Instituut TNO, Croesestraat 79,
Utrecht, Holland.

Aan allen, die aan de totstandkoming van dit proefschrift hebben meegewerkt

Voorwoord

Bij het gereedkomen van dit proefschrift tevens gekomen aan het einde van mijn academische en wetenschappelijke scholing is dit de juiste plaats en het beste moment om hen te noemen die deze vorming mogelijk maakten en ondersteunden.
Allereerst wil ik mijn dank betuigen aan U, Dr. A. Kaars Sijpesteijn, vooral voor het geduld dat U heeft gehad met deze niet – geborenwetenschappelijk werker. Mijn benaderingswijze gaf vele kansen op botsingen, maar Uw coöperatieve geest voorkwam deze gelukkig steeds weer. Uit onze vele gesprekken over de biochemische achtergronden van de natuurlijke resistentie en de overgevoeligheidsreactie is uiteindelijk dit onderzoek gegroeid. Ik ben er vast van overtuigd dat vele vragen in de fytopathologie opgelost kunnen worden met een meer fysiologische en biochemische benadering.
Het was een aangename ervaring, dat Prof. Dr. K. Verhoeff zo direct bereid was als promotor op te treden. Beste Koen, het is mij een eer je 'eersteling' te mogen zijn. Je grote belangstelling voor onderwerpen als het mijne en de wijze waarop jij dit proefschrift met voortvarendheid met mij hebt doorgewerkt, hebben een plezierige band geschapen.
Veel dank ben ik verschuldigd aan mijn promotor Prof. Dr. G. J. M. van der Kerk, die als directeur van het Organisch Chemisch Instituut TNO mij de gelegenheid gaf dit proefschrift te bewerken. Ik ben U vooral zeer dankbaar voor die gesprekken die wij mochten hebben over andere onderwerpen dan het direkte vakgebied. Uw belangstelling voor biologische problemen is bepaald opmerkelijk voor een organisch chemicus. Uw streven naar een interdisciplinaire benadering van wetenschappelijke problemen zou meer ingang moeten vinden in het wetenschappelijk onderzoek in Nederland.
De goede sfeer op de afdeling waar ik werkte was niet in de laatste plaats te danken aan mijn collega's Dr. J. C. Overeem en Drs. J. W. Vonk. Beste Jan en Hans, mijn grote dank voor het zes jaar uithouden met zo'n stuk onrust en mijn erkentelijkheid voor al hetgeen dat jullie voor mij deden.
Prof. Dr. J. Raa, Universiteit i Tromso, Norge, has been a guest worker at O.C.I. TNO between 1965 and 1968. His ideas and work initiate the research in the field of the biochemical background of the natural resistance. Dear Jan, your cooperation and friendship are gratefully acknowledged. Many thanks are due to Dr. Ray Lukens, Connecticut Agricultural Experiment Station for reading the English text.
Als kamergenoten hebben Drs. S. J. Dieleman en ik vele gesprekken gehad o.a. over de biochemische benadering van de resistentieproblemen en een daarvan mondde uit in de rekenmethode bij de lekproeven met radioactieve isotopen. Beste Stef, voor deze steun ben ik je zeer erkentelijk.
Veel dank aan Margreet van Vuuren, Toos Dekkers-van der Mark, Gerda Verkuil en Hanneke Jongerius-Wermenbol voor hun trouwe assistentie tijdens de duur van dit onderzoek verleend. Het was niet altijd even gemakkelijk omdat aanvankelijk positieve resultaten uitbleven. Gaarne wil ik de heer Van Kuijk hartelijk danken voor zijn uitstekende verzorging van de materialen in de broeikas.
Voorts dank ik de dames Leker, Janse-Leopold en Swierstra van het secretariaat voor het uittypen van het manuscript in al zijn stadia.
Dory Martens-Berg had de twijfelachtige eer om van mijn slechte handschrift een eerste type te maken. Ze heeft zich daarin geweldig geweerd en zich een groot improvisatrice getoond. Heel veel dank.
Mijn zwager Kees Nieuwenhuijzen verzorgde de

gehele lay-out van dit boekje en bewees daarmee dat ook van een dissertatie een mooi stuk typografie gemaakt kan worden. Beste Kees, ontzettend veel dank voor je aanbod om dit voor mij te doen en voor de grote hoeveelheid werk die je daarvoor moest verrichten.
Mijn vrouw verzorgde de na correctie, iets waarin ik bepaald niet uitblink. Een speciaal woord van waardering, lieve Els, is hier op zijn plaats.
De foto's en figuren werden verzorgd door Jan Minnaard. Rekening houdend met de krappe tijd die ik hem toemat heeft hij de taak glansrijk volbracht. Beste Jan, veel dank onder de spreuk 'adept-adopt-improve'.
Ir. Ietje Boukema (I.V.T.) en Ir. N. Hubbeling (I.P.O.) waren mij zeer behulpzaam bij het uitvoeren van inoculatie en determinatie proeven. Zonder hun samenwerking zou een dergelijk onderzoek onmogelijk zijn.
Voorts dank ik iedereen op het Organisch Chemisch Instituut TNO die op enigerlei wijze bijgedragen heeft aan de totstandkoming van dit proefschrift.
De Centrale Organisatie TNO is mij bij de voorbereiding en de uitgave van dit proefschrift in grote mate tegemoet gekomen.
Ik dank de directie van Gist-Brocades N.V. voor de vrijheid die mij gelaten werd in het laatste voorbereidingsstadium van mijn promotie.

Dit onderzoek werd gesteund door een subsidie van de Nationale Raad voor Landbouwkundig Onderzoek TNO.

Contents

A General introduction

The subject of this thesis is the natural resistance of tomato plants to *Cladosporium fulvum*. Before entering into details with regard to this host-parasite combination a more general survey of natural resistance to plant diseases is given. Terminology used in the present study is mostly derived from Wood (1967) and Van der Plank (1968).
In natural resistance one can quite generally distinguish two different situations between plant and fungus (*cf.* Kaars Sijpesteijn, 1969).

- 1) The fungus cannot attack the plant, because it simply has no tools for it. One might say there is no relation between plant and fungus. Kuc (1966) called this a 'zero-relationship'.
- 2) Alternatively plants can show resistance if they are able to resist the attack of a pathogen or to avoid the establishment of a pathogenic relation (Wood, 1967). In this case defense factors are required by the plant.

B Mechanisms of natural resistance

We will only deal here with the defense factors as meant under A 2. One can distinguish a number of mechanisms: the most important ones will be described here.

1 A rather passive defense mechanism is the built-in insensitivity for a *host-specific* toxin of the invading fungus. The toxin excreted by the penetrating hyphae of many Helminthosporium species damages the host cells of susceptible plants to such an extent that the fungus is able to develop more or less saprophytically. In certain cases, *e.g. Helminthosporium victoriae* on oats (Luke and Wheeler, 1955; Wheeler and Luke, 1963), *Helminthosporium carbonum* on corn (Scheffer and Ullstrup, 1965) and *Helminthosporium miyabeanus* on rice (Nakamura and Ishibashi, 1958) the structures of some of these pathotoxins have been partly unravelled and some of their physiological activities have been described. It is suggested that this type of compounds reacts with specific receptors in the membranes of the susceptible plants. The resistant cell membrane appears to lack the receptor or sensitive site since resistant tissue is not affected by the toxin (Samaddar and Scheffer, 1968). The toxin of *Helminthosporium maydis*, the causal fungus of the southern corn leaf blight, influences the respiratory activity of corn leaf mitochondria isolated from susceptible hybrids. In contrast, mitochondria from resistant plants are insensitive (Miller and Koeppe, 1971).
Isolates of such fungi which do not produce the pathotoxins cannot infect the plants, however, when in addition to the inoculum a pathotoxin is added, they become pathogenic (Joder and Scheffer, 1969). This suggests that resistance and susceptibility are determined by two properties, one of the plant: the sensitivity or insensitivity towards the toxic fungal products and the second of the fungus: the production of a host-specific pathotoxin.

2 The plants' condition is an important factor for many plant pathogenic fungi, some being able to infect only when the physiological condition of the plants or at least that of certain plant parts are suboptimal. A well-known example is the so-called *weak parasite Botrytis cinerea*. This fungus attacks plant part when

they are wounded or they have to use organic debris lying against living plants.
However, under some conditions, unwounded parts are infected (Jarvis, 1962; Verhoeff, 1970). Healthy tissue in optimal physiological conditions is, unwounded, fully resistant.

3 Another mechanism of resistance although until now rather theoretical, is the *inhibition of pectolytic enzymes*. Pectolytic enzymes play an important rôle in the disease syndrome of many plant diseases. If plants would be able to inhibit the macerating effects of pectolytic enzymes fungal infection in many cases would be unsuccessful. A recent study of Albersheim and Anderson (1971) gives supporting data for the rôle of pectolytic and cellulolytic enzyme inhibitors. Compounds with a structure like the phyto-hemagglutinins may be acting as such. Agents which are known to act as inhibitors of pectolysis are mostly of phenolic origin. Such compounds often arise as a result of infection. However, only little evidence for the rôle of pectolytic enzyme inhibitors in natural disease resistance has yet been presented (Wood, 1960; Wood, 1967).

4 The active formation of mechanical barriers by the so-called *post-infectional shift in the aromatic biosynthesis* in living tissue has been studied extensively by Rohringer and Samborski (1967). The aromatic metabolism in wheat is changed as a consequence of the invasion of the rust fungus. In some cases, as was demonstrated for resistant cucumbers infected with *Cladosporium cucumerinum* by Hijwegen (1963), lignin-like substances are produced around the site of penetration which form a mechanical barrier against the penetrating hyphae. Stahmann and coworkers (1966) observed the production of ethylene in diseased tissues. They suggested that ethylene is involved in the stimulation of peroxidase, phenylalanine lyase and polyphenoloxidase, enzymes which are all active in the production of lignin-like substances.

5 The next mechanism is based on the *phytoalexin theory* of Müller and Börger (1940).
A defense system is assumed to be built up by the production of inhibitory substances after infection. Such fungicidal or fungistatic compounds are called phytoalexins. Later Cruickshank (1963) greatly extended this approach working with different species of beans and peas. From inoculated pods of *Pisum sativum* and *Phaseolus vulgaris* he and his coworkers isolated two phytoalexins: pisatin and phaseollin.
The importance of phytoalexins in natural disease resistance still remains to be established. Müller (1959) and Cruickshank (1963) not only use in most instances unusual plant-fungus combinations, but apart from this none of the tissues they used – inner potato tuber tissue and inner epidermis of bean and pea pod – is ever parasitized in this way under field conditions. Thus they try to explain why non-parasites are not parasitic. In our opinion it is preferable to study the mechanism by which sometimes varieties of the same plant species are either resistant or susceptible to a certain pathogen. In the literature very little information is available to explain such a case in terms of phytoalexins. Bailey and Deverall (1971) working with varieties of *Phaseolus vulgaris* and physiological races of *Colletotrichum lindemuthianum* used a simple gene-

for-gene system in studying the rôle of phaseollin in this host-parasite combination. Their results are in contrast with the work of Cruickshank (1963) using seed cavities of bean pod. The data obtained from work with the seed cavities and that of work with hypocotyl are not correlating.
It was observed that salts of heavy metals such as $CuCl_2$ and $HgCl_2$ may also induce a tissue to produce phytoalexins. Thus the production of phytoalexins may be regarded as a more general response to stress conditions, arising both by the application on bean pod tissue of a droplet carrying spores of *Monilia fructicola* or a droplet containing $CuCl_2$. This stress condition obviously causes an alteration of cellular metabolism, leading to the extra formation of abnormal metabolic products. There is much reason to presume that also other abnormal substances are formed.

6 Finally a *hypersensitive reaction* frequently occurs as a resistance mechanism. Müller (1959) defines *hypersensitivity* as the premature dying of the infected tissue together with the inactivation or localization of the infectious agent. This process, sometimes called the necrogenic reaction, leads to a local necrosis to which the parasite is confined. The actual cause of this cell destruction is poorly understood. In a few cases (Raa and Kaars Sijpesteijn, 1968; Kaars Sijpesteijn, 1969) it has been suggested that the destruction of the plant cell membranes is involved. The fact that the development of non-obligate pathogens is suppressed is sometimes explained by the presence of naturally occurring or post-infectionally formed fungicides.
A well-studied example of a hypersensitive reaction followed by local necrosis is seen in the resistance of potato *(Solanum tuberosum)* inoculated with *Phytophthora infestans*. After inoculation of the epidermis cells of young leaves Tomiyama and coworkers (1967; 1967; 1971) made the following observations: a few minutes after the invasion of the hyphae the epidermal cells start to colour brown and die within an hour. This process is followed by complete desintegration of the cells around the site of penetration. Several biochemical changes are observed: higher oxygen uptake, appearance of mitochondrion-like vesicles in the vacuoles, higher peroxidase and polyphenoloxidase activity followed by the formation of lignin. These reactions are a normal response to biological, mechanical or chemical injury and are thus non-specific. In the susceptible potato, however, the plant tissue is not immediately killed by the pathogen which developes in the intercellular cavities.
As will be described later, the symptoms evoked by *Cladosporium fulvum* on resistant or susceptible tomato are in many ways comparable with those of *P. infestans* on potato.
Another example of a host-parasite relation involving a hypersensitive reaction is the combination apple *(Malus sylvestris)* – *Venturia inaequalis*. Noveroske and coworkers (1964) reported the formation of a post-infectionally formed fungicide. Later, this host-parasite relation was thoroughly investigated by Raa at our Institute (Raa, 1968a; Raa, 1968b; Raa and Kaars Sijpesteijn, 1968). The results can be shortly summarized as follows: The fungicide formed in homogenates of both resistant and susceptible apple leaves appears to be a direct oxidation product of the glucoside phloridzin, a compound present in both resistant and susceptible apple leaves, and is presumably a very unstable ortho-quinone (Raa and

Overeem, 1968; Overeem, 1969). In susceptible plants *V. inaequalis* is situated directly below the cuticle and establishes a parasitic relation without damaging the host tissue to such an extent that oxidation of phloridzin can take place. In resistant plants the cells react in a hypersensitive way. A rapid dying of the cells as well as oxidation of phloridzin occurs around the sites of penetration and the invading hyphae are killed by the fungicide formed. Raa (1968b) carried out experiments with dialysed culture filtrates of a particular strain of *V. inaequalis*. This preparation was able to evoke a reaction when applied to the leaves of resistant apple, but susceptible leaves were insensitive. Thus he suggests that in the resistant host the fungus triggers cell decompartmentalization which initiates the oxidation of phloridzin to the above mentioned fungicide which in its turn, kills the fungus. Thus the resistant host is selectively sensitive to toxic excretion products of the fungus. The implications of these findings have a much wider bearing, especially when one considers the genetics of such host-parasite relations.
In the next section the genetics of certain plant-parasite combinations will be discussed briefly. The promising results of this work at our Institute prompted us to investigate the host-parasite relation of tomato and the leaf mould fungus *Cladosporium fulvum* which appeared to have many features in common with apple scab.

C Genetics of natural resistance

Resistance of plants to fungi can be governed by one gene – monogenic resistance – or by many genes – polygenic resistance. In the latter case, many genes are acting jointly to give resistance.
Genetics of monogenic resistance has been studied far more intensely than genetics of polygenic resistance, presumably because it is much easier to assess. In the literature the term field resistance is also used instead of polygenic resistance.
Some examples of monogenic resistance are: cucumber to *Cladosporium cucumerinum;* potato to *Phytophthora infestans;* apple to *Venturia inaequalis;* oats to *Helminthosporium victoriae;* pea to *Fusarium oxysporum* and tomato to *Cladosporium fulvum*.
Each gene controls the production of a certain factor with a special function: the resistance mechanism required for that particular host-parasite interaction.
In many diseases resistance is governed by several genes each of which controls resistance to only certain so-called *physiological races* of the pathogen. Examples are the resistance of potato to *P. infestans* which is determined by at least nine genes, called R-genes (Black et al., 1953; Malcolmson and Black, 1966); tomato to *C. fulvum* (Day, 1968) and flax rust to *Melampsora lini* (Flor, 1942).
By breeding and introduction of new resistant varieties of host plants, new races of the pathogenic fungus sometimes emerge which are distinguished by a different pattern of pathogenicity towards host varieties. It is questionable whether such newly discovered races were really recently formed or whether these races were already existing and were merely selectively accumulated by the new resistant varieties of the host plant. New races can originate from nuclear fusion, formation of heterokaryons or from sexual reproduction.
Genetic studies with *M. lini*, the causal agent of rust in flax, led Flor (1942) to the gene-for-gene hypothesis. This hypothesis states that for each

gene which conditions a resistance reaction of the host there is a corresponding gene in the parasite which controls virulence or avirulence. Crossing experiments of Flor (1956), both with the plant and the fungus, gave considerable support for this idea. The concept was further elaborated by Person (1959) and Person and coworkers (1962). The gene-for-gene concept is now widely accepted in plant pathology. Since in these gene-for-gene systems one plant variety can be resistant to one race and susceptible to another race of the fungus, it is convenient to use the expressions compatible and incompatible to indicate the relation between the host variety and the physiological race in question. Thus in an incompatible combination the fungus cannot attack the host plant, whereas in compatible combinations a pathogenic relation is established.

Boone and Keitt (1957) studied the genetics of pathogenicity of *V. inaequalis* by crossing different physiological races. They could distinguish at least seven different genes for pathogenicity corresponding with seven hypothetical genes for resistance in the plant and therefore concluded to a gene-for-gene relationship.

For this disease Day changed the term genes for pathogenicity into genes for avirulence (*cf.* Fincham and Day, 1963). Work at our Institute provided evidence for a biochemical interpretation for the gene-for-gene relation in this disease (Raa, 1968 a, b; Raa and Kaars Sijpesteijn, 1968). Strong indications were obtained that the fungus excretes products selectively toxic to the incompatible plant; the hypothesis of Day that the genes for pathogenicity in fact are genes for avirulence was corroborated by these findings. It is assumed that the plant possesses selective receptors for the toxic product of incompatible pathogens; these receptors for the toxic product presumably are located in the membrane. In incompatible combinations of plant and fungus interaction of toxin and receptor triggers a hypersensitive reaction which is followed by necrosis. The resistance genes in the apple host would then control the presence of such receptor sites. In compatible combinations no such reactions take place because the toxin is not formed or the receptor is absent. In analogy to similar host-parasite relations earlier described for other diseases the complicated relation between host varieties of the tomato and physiological races of *C. fulvum* has been recognized by Day (1954) as a gene-for-gene relationship (see also Bailey, 1947 and Kooistra, 1964).

It occurred to us that the availability of several tomato varieties with different genes for resistance and of a number of physiological races of *C. fulvum* should provide an excellent tool for biochemical studies of this host-parasite relation.

In Chapter II the host-parasite relation tomato- *C. fulvum* will be considered in more detail and an outline of the present study will be given.

A Tomato leaf mould

Tomato leaf mould is caused by the hyphomycete (Fungi Imperfecti) *Cladosporium fulvum* Cooke, a facultative parasite. It is an economically important disease in glassshouse tomato crops (Smith et al., 1969) and in some areas in the open, where humid and high temperature conditions prevail. The disease was first described in 1883 by Cooke (1906) in England. It presumably originates from South-America, the homeland of the tomato.
The infection process can be described as follows: Germ tubes, arising from the conidia, penetrate through the stomata at the lower side of the leaves and procure lesions which in a compatible combination are followed by an extensive intercellular mycelial mat (*cf.* Persiel, 1967). Conidiophores emerge again through the stomata and produce a brown conidia. Sporulation is abundant at temperatures ranging from 20°–26° C. Only after cell death the hyphae do enter the cells. Yellow-coloured spots on the upper surface of the leaves (*cf.* Plate 1, Chapter III) are associated with death of the underlying palisade parenchyma, situated above the sporulating areas (Bond, 1938). Fruit infection is quite rare which may be due to the complete absence of stomata on the fruits.
Infection is strongly influenced by a number of external factors amongst which high temperature and excessive humidity play a predominant rôle. Optimum temperature for infection is about 22° C and optimum relative humidity is approx. 98% (*cf.* Butler and Jones, 1949).
Resistant plants respond to infection by a hypersensitive reaction, although not all resistance reactions are the same. A detailed description of the symptoms will be given in Chapter III.
Bond (1938) compared the behaviour of penetrating mycelium of *C. fulvum* in resistant and susceptible plants. In either case germination proceeds normally and a mycelium is formed. In the resistant leaf, however, this mycelium is very restricted, does not produce conidiophores and conidia and ultimately dies off. Some resistant varieties react on infection with an extended necrogenic reaction, whereas in others only a small number of cells is affected. The necrogenic spots finally dry out and turn brownish-yellow. The reaction of a certain tomato plant to the same physiological race changes with the external circumstances and depends among others on light intensity (Hubbeling, 1971). Low light intensity is held largely responsible for the lack of expression of the resistance which is sometimes observed.
C. fulvum can easily be cultivated on artificial media; frequently in these cultures spontaneous mutations occur, leading to changes in morphology of the mycelium as well as in the colour of the spores.

B Breeding for resistance

1 *Genetics of leaf mould resistance in tomato.* As early as 1933 Langford started with the breeding of leaf mould resistant tomato varieties. He found that all commercial tomato varieties used at that time were susceptible to several isolates of *C. fulvum*, with the exception of the variety Stirling Castle. This variety proved to be resistant to some isolates of *C. fulvum*, but susceptible to others. The resistance factor of Stirling Castle was later called the Cf 1 gene for resistance. This was the first time that physiological

specialization of races of *C. fulvum* was recognized. The discovery has had important implications for later tomato breeding programmes. It was already known that wild Lycopersicon species were fully resistant to all isolates of *C. fulvum;* these species, however, yield only very small fruits with no commercial value.

In 1939 the first resistant commercial tomato plant which was gained by crossing an existing commercial tomato variety with the wild *Lycopersicon pimpinellifolium*, was introduced. From one of such crosses the resistant tomato variety Vetomold emerged. Its resistance is based on one single dominant gene (Bailey and Langford, 1946). This gene was later called the Cf 2 gene for resistance. Soon after this success other Lycopersicon species were used in breeding programmes. New resistance factors were found in *L. hirsutum, L. peruvianum* var. *glabratum* and *L. glandulosum.* But soon after the introduction of the new resistant varieties resistance always appeared to be broken again, due to the appearance of new races of the fungus. A study of the pathogenicity of these 'new' races demonstrated clearly the existence of physiological specialization (Day, 1954; *cf.* Table I).

From the crosses with *L. pimpinellifolium* the conclusion was drawn that this species contains at least two dominant resistance factors, located on different chromosomes, each governing resistance against certain races of *C. fulvum.* These crosses produced the variety V 121, introduced in 1941 carrying the Cf 3 gene for resistance (Kooistra, 1964).

From 1940 onwards projects were initiated in Ontario and Massachusetts to transfer resistance from Lycopersicon species into commercial tomato varieties that were taxonomically more distantly related than *L. pimpinellifolium.* Several commercial varieties have been obtained from *L. hirsutum* (Guba, 1960). These varieties carry the Cf 4 gene for resistance (Kerr and Bailey, 1964). The genes Cf 1, Cf 2 and Cf 3 are located on different chromosomes (Barton et al., 1955) and the gene Cf 4 is supposed to be on the same chromosome as Cf 1 (*cf.* Kerr and Bailey, 1964). The dominant alleles of each of these determine resistance to certain races of the pathogen but they are quite ineffective towards other races. According to Quadt (1953) the two resistance genes from *L. pimpinellifolium* (Cf 2 and Cf 3) are not equivalent. One gene (Cf 2) was supposed to be a growth-restrictive gene while the other (Cf 3) was regarded to perform a necrogenic reaction. He stated that the necrogenic gene was hypostatic over the restrictive gene. Kerr and Bailey (1964) presume that resistance obtained from crosses with *L. hirsutum* and *L. hirsutum* var. *glabratum* is based on two dominant genes on two different chromosomes (Cf 2, Cf 4). Resistance from *L. peruvianum* is based on one dominant gene (Cf 4) (Bailey and Kerr, 1964).

Early in this breeding work some confusion existed about the designation of the resistance genes, but a new notation system was introduced by Day (1956). He based this system on the gene-for-gene hypothesis as put forward for the first time by Flor (1956).

Table I lists the relation between resistance factors and the races of the fungus (Day, 1956; Termohlen, 1960; Hubbeling, 1968). The absence of sporulation has been used as the only criterion for resistance. The varieties are indicated by the dominant resistance alleles present. In the literature on resistance of tomatoes to *C. fulvum* the recessive alleles are always

Table I Relation between the tomato varieties used as selective hosts and the physiological races of *C. fulvum*.
R: Resistant; S: Susceptible.

Tomato variety	Usual nomenclature	Genes for resistance (single set)	Physiological races of *Cladosporium fulvum*								
			Indices as given by Kooistra (1964) and Hubbeling (1968)								
			0	1	2	3	4	1.2	1.2.3.	2.3.4	1.2.4
			Presumed genes for avirulence								
			$A_1A_2A_3A_4$	$a_1A_2A_3A_4$	$A_1a_2A_3A_4$	$A_1A_2a_3A_4$	$A_1A_2A_3a_4$	$a_1a_2A_3A_4$	$a_1a_2a_3A_4$	$A_1a_2a_3a_4$	$a_1a_2A_3a_4$
Money-maker	cf	cf 1 cf 2 cf 3 cf 4	S	S	S	S	S	S	S	S	S
Leaf Mould Resister No. 1	Cf 1	Cf 1 cf 2 cf 3 cf 4	R	S	R	R	R	S	S	R	S
Vetomold	Cf 2	cf 1 Cf 2 cf 3 cf 4	R	R	S	R	R	S	S	S	S
V 121	Cf 3	cf 1 cf 2 Cf 3 cf 4	R	R	R	S	R	R	S	S	R
Purdue 135	Cf 4	cf 1 cf 2 cf 3 Cf 4	R	R	R	R	S	R	R	S	S
V 473	Cf 1 Cf 2	Cf 1 Cf 2 cf 3 cf 4	R	R	R	R	R	S	S	R	S
Vagabond	Cf 2 Cf 4	cf 1 Cf 2 cf 3 Cf 4	R	R	R	R	R	R	R	S	S

omitted. The same was done in the text of this dissertation. In Table I the full genotype for resistance (single set) is given als well. In 'resistant' varieties the leaves show chlorotic spots after infection; 'immune' varieties, however, are without any visible symptoms (Van der Plank, 1968). In Chapter III these disease symptoms will be discussed in detail. In this dissertation no distinction has been made between resistance and immunity as sometimes is done in the literature. The term immunity moreover has to be avoided in plant pathology, because of its use in animal pathology, where it has a different meaning.

2 *Physiological specialization of C. fulvum*
In the fungus at least 4 independent factors have been recognized which govern compatibility or incompatibility (*cf.* Table I).
Langford (1937) was the first to isolate different races of *C. fulvum* and he postulated that mutation was the source of these new physiological races. Later Bailey (1950) came to the same conclusion. The hypothesis of stepwise mutations has been strengthened by the work of Day (1957), who, by means of ultraviolet irradiation, induced a mutation of race 0 into race 2 (indices as used by Kooistra and Hubbeling, see Table I). This race 2 attacks the variety Vetomold which is resistant to race 0. The fact that Day's mutation was induced in a red-pigmented isolate eliminated contamination of his strain as a source of error. As we shall see later this result is in favour of the theory as an explanation for physiological specialization.
C. fulvum is propagated asexually and only in one case (Ciferro, 1954) the finding of a perfect stage has been reported. If this perfect stage exists, sexual recombination could also be a source of new races of the fungus.
Day (1957) did not succeed in producing heterokaryons of *C. fulvum;* only the tip cells of the hyphae contain more than one nucleus. Nuclear fusion should also be considered as a possibility for the formation of new races of the fungus. Hyphal fusion has been reported for *C. fulvum* and this may give rise to the idea of exchange of nuclear material (De Vries, 1952 and Day, 1957). De Vries (1952) supposed that pycnospores play a rôle in the mutation to virulence, but no decisive evidence exists on this point.
Whatever may be the source of physiological races of *C. fulvum*, their existence has been proved. Inoculation tests have revealed that by cultivation of these races in vitro their varietal specifity remains stable.

C Hypothesis on the biochemical explanation of the natural resistance of tomato to C. fulvum

1 *Earlier work.* Only little work has been done on the nature of the natural resistance of tomato to *C. fulvum.* Bailey and Lowther (1962) and Lowther (1964) carried out careful analytical studies to investigate whether chemical composition and nutritional condition of the host affected influence on its response to the fungus. These studies support only in part the hypothesis that levels and kinds of nutrients in host tissues are of predominant importance for the successful development of the pathogen. Studies of Ghabrial and Pirone (1966) on the nutritional conditions of the plant could not explain the resistance to leaf mould. In general, a suboptimal growth condition of the tomato appeared

Table II Cross relation between two tomato varieties and two races of *C. fulvum* with regard to their resistance to *C. fulvum*. S: Susceptibility; R: Resistance.

	Physiological race of *C. fulvum*	
	1	2
Tomato variety Cf 1	S	R
Tomato variety Cf 2	R	S

to decrease resistance to incompatible races of *C. fulvum*, but in tomato plants growing under optimal conditions no conclusive differences in chemical composition could be detected to explain resistance.

Next to the negative results of the work described above, it is obvious that such a hypothesis for the natural resistance of tomato to *C. fulvum* can never explain the gene-for-gene relation. The cross relationship as given in Table II, which illustrates the basic principle underlying the gene-for-gene relation, makes that clear. The physiological races 1 and 2 are distinguished by their pathogenic behaviour. Equally the tomato varieties carrying the Cf 1 and Cf 2 genes for resistance differ in their resistance to different races of *C. fulvum*. It is obvious from Table II that a difference in the chemical composition of the tomato varieties can never explain resistance to one physiological race and at the same time susceptibility to another race. Since any biochemical explanation of the natural resistance mechanism must be in accordance with the genetic data, one must conclude that factors both from the host and from the pathogen decide together over resistance or susceptibility. The present hypothesis is based on this principle.

2 *The present hypothesis.* As described in Chapter I Raa (1968a) obtained strong indication that in incompatible combinations of apple and *V. inaequalis* a selective toxin of the fungus initiates the production of a fungicidal compound in the host plant. Furthermore, the hypothesis was developed that in the gene-for-gene relation of this disease different avirulence genes in the pathogen control the production of a variety of host-specific toxins each capable of evoking a hypersensitive reaction on the

appropriate apple plant (Raa and Kaars Sijpesteijn, 1968). The resistance genes of the host plant are supposed to be responsible for the production of selective receptors. Each receptor corresponds with one avirulence gene in such a way that together they determine the hypersensitive reaction.
Kaars Sijpesteijn (1969) proposed that the same principle might underly the host-parasite relation in leaf mould of tomato and in other plant-parasite combinations with a gene-for-gene relation system. This would imply the occurrence of four avirulence genes, called A-genes, in *C. fulvum* corresponding to the four resistance genes (Cf-genes) in the tomato. Thus any tomato plant carrying the dominant allele Cf 1 is resistant to any physiological race carrying the avirulence allele A_1. According to this hypothesis the alleles A_1, A_2. A_3 and A_4 of the four avirulence genes govern the production of selective toxins and consequently the initiation of the hypersensitive reaction in tomato plant, in contrast to the corresponding alleles a_1, a_2, a_3 and a_4. In Table I the presumed genotype with regard to avirulence genes is given for the races of the fungus used in the present study, or in other words the genotype for toxin production. In a previous publication (Van Dijkman and Kaars Sijpesteijn, 1971) we have already introduced this new system to designate the physiological races of the fungus.
This hypothesis may explain the hypersensitive reaction. For a facultative parasite, however, a hypersensitive reaction in itself does not explain cessation of fungal growth. In the case of resistance of apple to *V. inaequalis*, fungicidal compounds arise from the oxidation of phloridzin. Kern (1952) suggested that in the resistance of tomato plant to *C. fulvum*, the fungicidal constituent tomatine, a glycosidic steroid-alkaloid (Fontaine et al., 1948), might play a rôle. This phenomenon led us to investigate if this compound is of importance in the resistance of tomato plants to *C. fulvum*. Tomato leaf mould is an attractive object for studying the gene-for-gene relationship in plant disease because of the availability of seeds of different tomato varieties as well as of the different races of *C. fulvum*.
In this study it is attempted to present evidence for the above hypothesis.

D Outline of the present study

The purpose of this study was to investigate the biochemical background of the natural resistance of tomato to *C. fulvum*. On the basis of the hypothesis presented in this chapter we tried to obtain evidence by means of model experiments whether metabolic excretion products of the fungus play a rôle in the hypersensitive reaction of tomato tissue.
Chapter III deals with some physiological experiments with the various races of *C. fulvum*. The results of inoculation experiments and a description of the different symptoms on the varietal host plants is given. Chapter IV presents an account of the model experiments with the high molecular weight excretion products of *C. fulvum*. Leakage of ^{32}P from labelled leaf discs on infiltration with these excretion products was compared in compatible and incompatible combinations. Properties of these extracellular excretion products are given. Chapter V deals with the fungicidal properties of tomatine and its possible rôle in the cessation of growth following the hypersensitive reaction. In Chapter VI the

electrophoretical differences of the physiological races of *C. fulvum* are described. A general discussion of the results as well as a biochemical interpretation of the genetics of the host-parasite relation between tomato and *C. fulvum* is presented in Chapter VII.

Chapter III

Cladosporium fulvum, growth in vitro and in vivo

A Introduction

1 *Nutritional requirements*

C. fulvum is a comparatively slow growing fungus, although it can be readily cultivated on several complex media. Lowther (1964) reports that three isolates of the fungus were able to grow well on the carbohydrates saccharose, fructose or glucose with only slight variation in mycelial dry weight production. As nitrogen source all amino acids except cysteine supported growth, although with little differences in mycelium dry weight. The lack of growth on media containing cysteine may be due to fungitoxicity of this compound. Lowther (1964) claimed that pathogenicity of the fungus was changed by growth on different nitrogen sources, but these findings could not be confirmed by Ghabrial and Pirone (1966). The intercellular growth of *C. fulvum* after penetrating through the stomata could possibly suggest that the fungus is unable to degrade cell walls, but no information is available on its cellulolytic and pectolytic properties.

The influence of the pH on growth has not been investigated.

Obviously more knowledge on the nutritional requirements and optimum conditions for growth was required for our studies.

2 *Inoculation of different tomato varieties with races of C. fulvum*

Inoculations and re-isolations were performed, firstly to retain pathogenic characteristics of the fungus, which tend to change on artificial media. Secondly the pathogenic pattern was checked on the appropriate varietal host plants. Moreover, the symptoms of the hypersensitive resistance reaction governed by the resistance genes of the tomato varieties were studied. Symptoms have been described earlier but not for all resistance genes in a summarizing way. The appearance of chlorotic spots in the hypersensitive reaction, with chlorosis taking place some cell layers beyond the invading hyphae, is of basic interest in the biochemistry of this disease. Chlorophyll breakdown occurs without visible damage to the cells. Apparently the host cells several layers distant from the fungus are disturbed in such a way that the enzyme chlorophyllase, which decomposes chlorophyll, is released.

B Materials and methods

1 *Fungal races and plant varieties*

Nine different physiological races of *C. fulvum* were obtained from Ir. N. Hubbeling of the Institute for Phytopathological Research (I.P.O.) in Wageningen. These have been listed in Table I, Chapter II. Of each race monoconidial isolates were prepared.

Seeds of the different tomato varieties, each of which being homozygous for their genes for resistance were obtained from the Institute for Horticultural Plant Breeding (I.V.T.) in Wageningen. Table I from Chapter II gives the true genotypes of the plants in the haploid state; throughout this thesis only the dominant alleles for resistance are indicated as usual in the pertinent literature. Plants were grown in the glasshouse with a 16-hour daylight period at a temperature of 20–24 °C. Nearly in all experiments healthy mature leaves of approximately the same age were used to avoid the influence of scenescene in our experiments.

2 *Techniques*

a *Cultivation of C. fulvum.* The monoconidial isolates and the re-isolates were kept on potato dextrose-agar (PDA) slants at 24°C in the dark. Other media, as malt agar, V-8 juice agar and oat meal were tried as well. Stock cultures were transferred every three to four weeks to freshly prepared PDA slants in an attempt to maintain a standard culture. Only conidia were used for inoculation purposes. On making transfers care was taken to avoid culturing spontaneous mutations of off-coloured conidia and sterile mycelial puffs. Shake cultures were grown at 24°C in the following protein free medium containing unless otherwise stated, 2% glucose, 0.2% casamino acids (Difco, technical grade), 2 ppm $ZnCl_2$, 2 ppm $MnSO_4.4H_2O$, 0.15% $MgSO_4.7H_2O$, 0.011% KCl, 0.0025 M K_2HPO_4/KH_2PO_4 (pH 6) in tap water.
Sterilization was carried out by autoclaving, at 120°C during 15 min. Inoculation of shake cultures was executed by adding conidia from one culture tube (approx. 4 weeks old) to the medium. The cultures were incubated for 3–4 weeks at 24°C. The pH of the sterile basal medium lays within the range 6.0–6.5. Flasks of 1 liter containing 125 ml of medium were used to assure an ample supply of oxygen for growth. To study the effect of various nitrogen or carbon sources the corresponding compound from the basal medium was replaced. Mycelium was filtered off and dried over P_2O_5 in vacuo or in an oven at 110°C and subsequently weighed.

b *Determination of enzyme activities.* Cellulase activity was estimated by the ability of *C. fulvum* to grow on cellulose as the sole carbon source as well as its ability to decompose cellophane. Pectinase activity of the freeze-dried void-volume fraction (*cf.* Chapter IV) of race $A_1A_2A_3A_4$ of *C. fulvum* was determined by measuring the rate of decrease of viscosity of solutions containing 0.8% pectin in phosphate buffer (0.01 M, pH 5.3).
Viscosity was measured at 25°C with an Oswald type viscosimeter which had a straight capillary, forming a bulb of 6 ml volume at a distance of 11 cm from the efflux end. The efflux times of the test solution from the bulb were recorded and the specific viscosity (η spec) was calculated with the formula:

$$\eta \text{ spec} = \frac{t_t - t_c}{t_c}$$

t_t = efflux time of test solution
t_c = efflux time of a control solution without pectin

The fungal pectinase preparation was obtained from Th. Schuchardt, München; a 1% solution of this enzyme was applied.

c *Inoculations* were performed by spraying a dense conidial suspension over the plants in a moist chamber or in a glasshouse under high humidity conditions at 20–22°C.
The plants had at least three leaves when inoculated. The varietal hosts (see Table I, Chapter II) were used for inoculations except for attempts to recover the pathogenicity of some races of *C. fulvum*. For the latter purpose the tomato variety Philippine was employed because it was very susceptible to all known races of *C. fulvum*.

d *Re-isolations* were made to check the pathogenic stability of the various physiological races, by taking some conidia from the sporulating area of the

infected leaf on a wet transferring loop and placing them on a PDA plate. As soon as small fungal colonies of *C. fulvum* carrying spores appeared these were transferred to a new PDA plate by the same technique. The cultures were checked for bacterial contamination by inoculating fungal fragments on peptone-glucose agar (PGA) slants. Conidia from pure cultures were freeze-dried in skim milk and stored in a refrigerator.

C Results

1 *Growth experiments with C. fulvum in vitro*
Good growth and sporulation were obtained on the following media: potato dextrose agar (PDA), Czapek-Dox agar, oat meal agar, malt agar and V-8 juice agar. PDA slants were used throughout this study to maintain the cultures, because this medium meets the requirements the best. The pH of the media varies within the range 6–6.5 after autoclaving.

a *Carbon sources.* Growth of *C. fulvum* on different carbon sources was tested on agar plates as well as in shake cultures with the basal medium. Glucose as carbon source was replaced by the compounds mentioned in Table I. The pH of the media ranged from 6.3 to 7.1. The flasks were inoculated with equal amounts of spores.
The results show that of the carbon sources tested only glucose, saccharose and fructose give the highest mycelum dry weights. Experiments on agar corroborated these results.

b *Nitrogen sources.* Similar experiments were undertaken to establish the influence of different

Table I Influence of the carbon source (1 %) on mycelium dry weight of *C. fulvum* race $A_1A_2A_3A_4$ after three weeks incubation at 24° C. Experiments in triplicate. Initial pH 6.5.

Carbon source (1 %)	*g mycelium dry wt.*
none	< 0.05
glucose	1.38
saccharose	1.44
fructose	1.30
galactose	0.48
cellulose	< 0.05
starch	< 0.05
pectin	0.14
galacturonic acid	0.68
malic acid	< 0.05
succinic acid	< 0.05

Table II Influence of the N-source (1 %, w/v) on mycelium dry weight of *C. fulvum* race $a_1a_2A_3a_4$ after three weeks incubation at 24° C. Initial pH 6.5. Experiments in triplicate.

Nitrogen source	*g mycelium dry wt.*
casamino acids	1.04
KNO_3	0.83
NH_4Cl	0.68
NH_4NO_3	0.32
l-alanine	0.52

nitrogen sources on the mycelium dry weight production. The results are tabulated in Table II. Experiments on agar confirmed these results.

c *Optimum pH for growth of C. fulvum.* PDA plates with different pH values ranging from 3.5 to 8.0, measured after autoclaving, were inoculated in the centre with conidia of *C. fulvum* race $A_1A_2A_3A_4$ and $a_1a_2A_3a_4$. After three weeks it appeared that in the pH 5–6.5 area maximum growth was obtained. The influence of pH on growth of *C. fulvum* race $a_1a_2a_3A_4$ was investigated by inoculating 1 l flasks with an equal amount of spores per flask (Table III). The pH of the basal medium was measured after autoclaving as well as before harvesting the mycelial fragments at the end of the incubation period. These data showed that optimal growth was procured at pH 5–6.

2 *Enzymatic tests with culture filtrates of C. fulvum*

a *Cellulase.* The culture filtrates were tested on their ability to decompose cellulose, the major constituent of the plant cell wall. It was demonstrated above that growth with cellulose as the sole carbon source was very poor. Sterilized cellophane was incubated for three weeks in a shake culture containing the complete basal medium but no loss of mechanical strength was observed. To investigate whether a growing mycelium mat of *C. fulvum* can hydrolyze cellulose, spores of *C. fulvum* race $A_1A_2A_3A_4$ were seeded on a PDA plate covered with a thin sheet of sterilized cellophane. The fungus formed a normal round colony and obviously the nutrients passed the cellophane membrane. After 6 and 12 weeks of incubation, the mechanical strength of the cellophane was judged by tearing the sheet by hand comparing them with the strength

Table III Influence of the pH on the mycelium dry weight production by *C. fulvum* race $a_1a_2a_3A_4$ after three weeks incubation at 24° C.

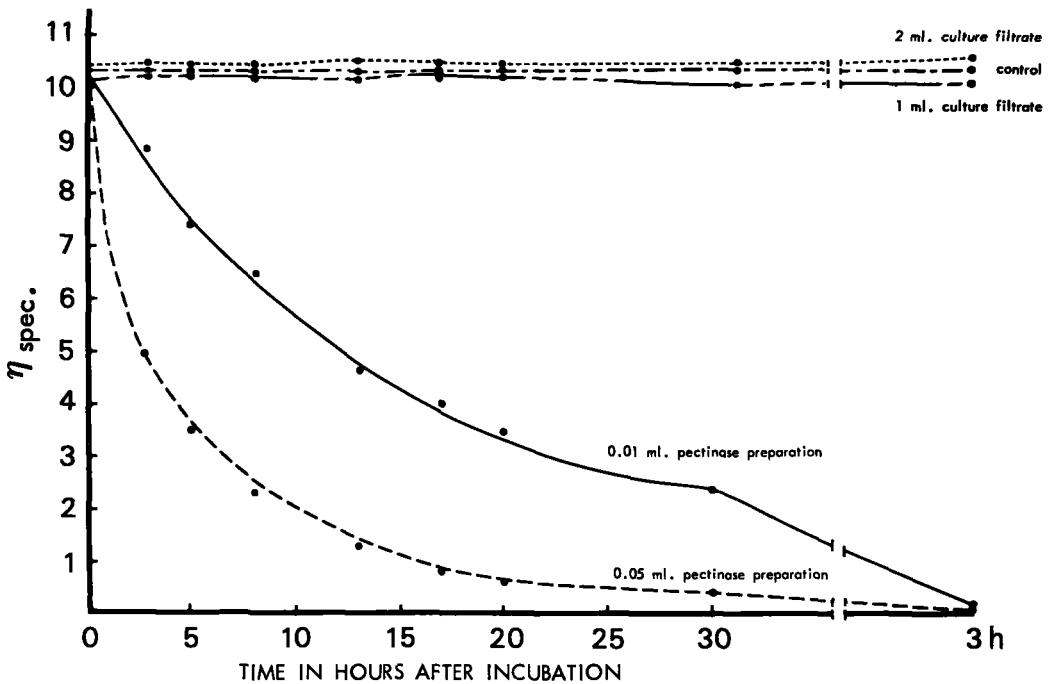
Initial pH	*Final pH*	*g mycelium dry wt.*
3.5	3.4	0.1
4.0	4.3	0.2
4.5	6.0	0.25
5.0	8.0	0.6
5.5	8.0	0.4
6.0	8.0	0.4
6.5	7.9	0.4
7.0	8.1	0.3
7.5	8.2	0.4
8.0	8.2	0.3
8.5	8.2	< 0.05

of a sheet of cellophane from a non-inoculated plate. There was no perceptible difference in strength between either sheet of cellophane. Staining with zinc-chlor-iodide solution (Rawlins and Takashashi, 1952; Jensen, 1962) which colours undecomposed cellulose blue and decomposition products red, agreed with these results.
From these experiments it appeared that the fungus does not produce any cellulase.

b *Pectinase.* Pectinase activity of the undiluted and unpurified culture filtrate was compared with the activity of a commercial pectinase preparation. Fig. 1 illustrates that the culture filtrate does not affect the viscosity of a 0.8% pectin solution.
Since the culture filtrate did not show any pectinase activity we tried to demonstrate pectinase activity in a lyophilized culture filtrate of the fungus. Following dialysis 375 ml culture filtrate yielded 1.13 g of dry powder; the preparation contained 21% protein as measured with the Folin method. Fifty mg of this powder dissolved in 10 ml 0.01 M phosphate buffer (pH 6) did not show any pectinase activity in the test method described before. Only little growth was observed on pectine as the sole carbon source. This growth is probably due to impurities of the commercial pectin preparation.

3 *Growth in vivo*
In this section results are recorded of a series of inoculation experiments carried out in cooperation with Ir. Ietje Boukema at the Institute for Horticultural Plantbreeding. They are illustrated in Plate 1–8.
Plate 1 shows a heavily infected leaf of the variety Moneymaker (cf, no genes for resistance) 14 days after inoculation with conidiospores of *C. fulvum*

Fig. 1 The effect of unpurified culture filtrate and of a commercial pectinase preparation on the viscosity of a 0.8% pectin solution. Incubation in 0.01 M phosphate buffer pH 5.3 at 25° C.



.....	25 ml of 0.8% pectin solution incubated with 0.01 ml of commercial pectinase preparation.
—·—·—·—	idem but with 0.05 ml of the commercial pectinase preparation.
— — — —	25 ml of 0.8% pectin solution incubated with 1 ml of the dialyzed culture filtrate of *C. fulvum* $A_1A_2A_3A_4$.
———	idem but with 2 ml of the dialyzed culture filtrate of *C. fulvum* $A_1A_2A_3A_4$.
- - - - - -	buffer control, 0.8% pectin solution incubated with 1 ml of buffer solution.

race $A_1A_2A_3A_4$. In general, symptoms develop between 10–14 days after inoculation. The brown-velvet areas in the right center part are abundant sporulating patches. The leaf is not yet as chlorotic as the leaves on the other plates (Plates 2–8), which were also made 14 days after inoculation.
In resistant leaves chlorosis takes place much earlier than in susceptible ones. In the compatible combination chlorosis does not start before the life cycle of the fungus is completed by producing new conidiospores.
Plate 2 gives the results of the inoculation of the variety Leaf Mould Resister No. 1 (Cf 1) with an incompatible race of *C. fulvum* ($A_1a_2a_3a_4$). The leaf on the left shows the earlier signs of a resistance reaction 14 days after the inoculation. The leaf on the right is of the same variety but in a more final stage of the resistance reaction. The first symptoms appear as chlorotic spots which enlarge gradually; this is followed by drying out of the local lesion. After some days the fungus has disappeared.
As a result of inoculation of the variety Leaf Mould Resister No. 1 (Cf 1) with an incompatible race of *C. fulvum*, some sterile mycelium can be formed on the lower side of the leaf as illustrated by Plate 3. These mycelial puffs disappear when the lesion becomes necrotic (right leaf Plate 2). No mycelium could be detected in these spots.
Plate 4 illustrates the reaction of a so-called fully resistant variety (Vetomold, Cf 2) to fungal races carrying the avirulence allele A_2. It is not clear whether the small chlorotic dots in the intervenal areas are the sites of the resistance reaction. By normal light microscopy no mycelium could be detected in cross sections through the spots stained with cotton blue in lactophenol.
The tomato varieties carrying the genes for resistance Cf 2 and Cf 4 react similarly upon inoculation with an incompatible race. Under normal glasshouse conditions no symptoms became visible in incompatible combinations involving the Cf 2 or Cf 4 gene for resistance. Only under optimal conditions did some symptoms appear.
Plate 5 shows the symptoms of the resistance reaction governed by the Cf 3 gene for resistance. The variety used, V 121, reacts with relatively large chlorotic spots in comparison with the symptoms developing after inoculation with incompatible races on plants with the Cf 2 and/or Cf 4 genes. The chlorotic resistance spots of the Cf 1 plants are, however, even larger. The combination of the two genes Cf 1 and Cf 3 in the variety 59 R gives much smaller chlorotic lesions upon inoculation with incompatible races than the spots governed by each gene separately. This is shown in Plate 6.
Plate 7 shows a close-up of an intervenal area of a leaf of the variety Purdue 135. This Cf 4 plant was inoculated with a very dense spore suspension of an incompatible race of *C. fulvum* ($a_1a_2a_3A_4$). The necrotic spots on the lower side of the leaf were clearly visible. No mycelium could be detected in microscopical preparations of the necrotic areas. A fully resistant plant is illustrated on Plate 8. This variety, Vagabond, with the genes Cf 2 and Cf 4, inoculated with a dense spore suspension of an incompatible race of *C. fulvum* ($A_1A_2A_3A_4$) shows complete resistance. No visible symptoms could be observed whilst the inoculation and incubation were carried out under the same circumstances and conditions as with the other plants in this experiment.
The pH of the leaves of the different tomato varieties used was determined by placing the

Plate 1 Tomato variety Moneymaker (cf) inoculated with a compatible race of *C. fulvum*, $A_1A_2A_3A_4$. The olive-green or brown-velvet areas are the sites of sporulation.

Plate 2 Tomato variety Leaf Mould Resister No. 1 (Cf 1) inoculated with an incompatible race of *C. fulvum*, $A_1a_2a_3a_4$. The left leaf gives early symptoms of a resistance reaction; the right leaf shows the final stage of such a reaction.

Plate 3 The lower side of leaves of the tomato variety Leaf Mould Resister No. 1 (Cf 1) inoculated with an incompatible race of *C. fulvum*, $A_1A_2A_3A_4$. Small sterile mycelial puffs are formed which disappear as the stage is reached as given in Plate 2 right leaf.

Plate 4 Reactions of the variety Vetomold (Cf 2) upon inoculation with an incompatible race of *C. fulvum*, $A_1A_2A_3a_4$. This variety is fully resistant. The very small chlorotic spots are presumably the sites of a resistance reaction.

Plate 5 The symptoms of the resistance reaction governed by the Cf 3 gene for resistance from the tomato variety V 121. *C. fulvum* race $a_1a_2A_3a_4$.

Plate 6 Local lesions of the variety 59 R (Cf 1 Cf 3) inoculated with *C. fulvum* race $a_1a_2A_3a_4$.

Plate 7 Close-up of an intervenal area of the tomato variety Purdue 135 (Cf 4) inoculated with an incompatible race of *C. fulvum*, $a_1a_2a_3A_4$.

Plate 8 Leaves of the variety Vagabond (Cf 2 Cf 4) inoculated at the same time after spraying the spores as the leaves shown on the other plates. No local lesions were visible. The inoculation was carried out with spores of the incompatible race $A_1A_2A_3A_4$.

electrodes of a Beckman Model N pH meter in the leaf homogenate produced by grinding leaf tissue in a mortar. The three youngest leaves from the top had a higher pH than older leaves. The highest pH for young leaves of the variety Moneymaker was 6.7, the lowest pH for the variety V 121 was pH 6.2. Older leaves of these varieties had a somewhat lower pH (about 6.1–6.2).

4 *Re-isolation of physiological races of C. fulvum*
For all fungal races used in the present experiments, re-isolations were carried out after inoculations upon a suitable host. As tomato variety the completely susceptible variety Philippine (cf), a variety on which all races *C. fulvum* show even better growth than on the variety Moneymaker, was used. After 10–12 days, spores were taken from the infected areas and seeded on PDA. The newly isolated strains were again inoculated on the differential hosts to verify their pathogenicity.
The colour and colony shape is varying from race to race. Even when their genetic code for avirulence is identical, differences in colour and colony form occur. In the course of this work *C. fulvum* race $A_1a_2A_3A_4$ lost ability to produce spores. The puff-like structures are puffs of sterile mycelium.

5 *Microscopical observations of infected tissue*
Cross sections through an infected area of a susceptible plant inoculated with a compatible race, stained with cotton blue in lactophenol, revealed a dense mycelial mat in the intercellular cavities of the palisade parenchyma. Until approx. 10 days after inoculation the cells were not desintegrated, but after 12–14 days, when the mycelium is already producing conidia, desintegration started, as shown by a strong chlorosis and a drying-out of the infected spots. In a successful infection the whole leaf dries out and in the final stage it is completely covered with dense masses of conidia.
These reactions vary somewhat for different varieties, depending on the virulence of the fungus and presumably also by the presence of polygenic resistance in some tomato varieties of the Cf 1 type.
Sections through local brown-dry necrogenic spots from leaves of tomatoes inoculated with incompatible races of *C. fulvum* did not show any mycelium. Only in the Cf 1 Cf 3 plants a sparse mycelium could be seen after staining. When the infected areas become necrotic the mycelium vanishes.
In sections of the yellowish pin-pointlike areas from Cf 2 and Cf 4 plants some hyphae only could be seen after staining. In most cases mycelium-like structures were found, but only a minor part stained with cotton blue in lactophenol, the hyphae presumably being already dead. It was observed that the chlorotic areas in the leaves were much larger than the tissue invaded with mycelium; this was very clear with the Cf 1 and Cf 3 tomatoes.

D Discussion

The findings of Lowther (1964) that good growth of *C. fulvum* was obtained on glucose, saccharose and fructose were confirmed. The other carbohydrates, tabulated in Table I, allow little or no growth. Of the nitrogen sources tested casamino acids provide the best growth, followed by KNO_3. Maximum growth in vitro was obtained in the pH range 5–6.5, whereas the pH's in the plants vary from 6.1–6.7 depending on the age of the leaves.

The inability of *C. fulvum* to produce cellulase or pectinase, is in line with its mode of penetration into the leaf of the tomato plant. The fungus cannot grow on cellulose or pectine; thus in compatible combinations the fungus must grow on the compounds leaking out of the intact cells into the intercellular cavities or on the contents of the destroyed cells.
Macroscopical observations revealed a much weaker hypersensitive reaction of the Cf 1 plants than of plants carrying the Cf 2, Cf 3 or Cf 4 alleles. The Cf 1 plants upon inoculation with an incompatible race react with chlorotic areas without a sharp boundary, whereas plants carrying the Cf 2 and/or Cf 4 alleles for resistance react with a more restricted pin-point spot (Hubbeling, 1966; Persiel, 1967). Brown-dry areas are seen in the centre. Upon inoculation with an incompatible race Cf 3 plants show a reaction intermediate between that of Cf 1 and Cf 2 or Cf 4. Observing the reaction of Cf 1 plants upon inoculation with either compatible or incompatible races of *C. fulvum*, the difference in the reactions, although distinct, is not found to be outstanding. It is assumed that plants carrying the Cf 1 allele possess also the so called horizontal resistance, governed by a set of other genes. This presumed polygenic resistance is at present under investigation at the Institute for Horticultural Plantbreeding in Wageningen. Also the influence of light intensity on the expression of the Cf 1 allele (Hubbeling, 1971) is not yet well understood. When plants are grown under conditions of low light intensity a weaker resistance reaction is found.
Combinations of the resistance alleles Cf 1 with Cf 2 or Cf 4, obtained in several breeding stations in Canada and Holland, revealed that the symptoms of the resistance reaction governed by the Cf 1 allele alone, are suppressed when the Cf 2 or Cf 4 allele is present as well. The Cf 2 allele and Cf 4 allele are epistatic over the Cf 1 allele (Persiel, 1967). Combinations of the Cf 3 allele with either Cf 2 or Cf 4 revealed that the latter are also epistatic over the former.
In tomato varieties with the Cf 1 and Cf 3 alleles for resistance combined, the resistance reactions are quite different compared to those which contain these alleles separately. There appears to be a cumulative effect, whereas the combination of the Cf 2 and Cf 4 alleles in one variety reacts substantially in the same way as these two alleles separately.
The observation that the mycelium was not found in the necrotic areas of the resistant plant raises the question of the cessation of growth in the incompatible host plant. However, we know that the hyphae penetrate through the stomata in the intercellular cavities and that the fungus is able to live on dead tomato plant tissue. In sections through the chlorotic areas hardly any mycelium could be detected. In Chapter Va more thorough discussion concerning the fate of the fungus following the hypersensitive reaction will be given.
To retain the virulence of our cultures, inoculations and re-isolations were made from time to time. The pathogenicity was checked on the appropriate hosts and, except in the case of the race $A_1a_2A_3A_4$ pathogenicity could always be recovered.

E Summary

Chapter III deals with experiments in vitro and in vivo with *C. fulvum*. The nutritional requirements of

C. fulvum were investigated and different carbon and nitrogen sources were tested. Glucose, saccharose and fructose provide good mycelium dry weights, whereas practically no growth was observed on starch, cellulose and pectin. As nitrogen source vitamin-free casamino acids appeared to be most suitable. The pH optimum is about 6. There was no activity of cellulase and pectinase observed in the culture filtrates.
Inoculation experiments were described with the various tomato varieties, all resistance genes being involved. The results have been recorded on colour plates and are briefly discussed.

Chapter IV

Membrane leakage in incompatible combinations of tomato and Cladosporium fulvum

A Introduction

The results from the experiments with incompatible combinations of apple and *Venturia inaequalis* described in Chapter I (Raa, 1968b; Raa and Kaars Sijpesteijn, 1968) suggest that toxic compounds excreted by the fungus disturb the cell structure and evoke a complete decompartmentalization of the cell in the surroundings of the penetrating hyphae. Raa (1968b) deducted this from his experiments in which he placed leaves of a susceptible and of a resistant variety of apple into a dialyzed culture filtrate of *V. inaequalis*. The leaves resistant to this race of Venturia wilted within a short time in contrast to the susceptible leaves. Apparently, the turgor of the companion cells around the xylem vessels loose their rigidity and collapse resulting in a wilted leaf. The suggestion was put forward that the active high molecular weight products from the fungus are proteins reacting specifically with certain receptors in the host plant (Kaars Sijpesteijn, 1969). Since the gene-for-gene relation between apple and *V. inaequalis* shows much similarity to that of the tomato-*C. fulvum* system we have tried to demonstrate a reaction of the same kind with *C. fulvum* preparations and incompatible and compatible combinations of tomato tissue and *C. fulvum*.

In Chapter I and II the hypothesis has been brought forward that excretion products of an incompatible *V. inaequalis* can evoke a reaction similar to the hypersensitive reaction in vivo. The disturbtion of the cell structure by specific compounds of the incompatible race leads to chlorosis and necrosis of the leaf-tissue. Translating this for the host-parasite combination tomato-*C. fulvum* it implicates the occurrence of four avirulence genes (A) in the haploid fungus corresponding to the four resistance genes (Cf) in the tomato in such a way that for instance any tomato carrying the dominant allele Cf 1 is resistant to any physiological race carrying the avirulence allele A_1. According to this hypothesis the alleles A_1, A_2, A_3 and A_4 of the four avirulence genes effect the production and excretion of four different toxic compounds by the fungus, each of which evokes leakage of cell constituents, and as a consequence a hypersensitive reaction, in the host plant with the corresponding Cf allele. On the other hand a tomato plant will be susceptible to a race if no A allele corresponding to a Cf allele is present. In Table I from Chapter II the hypothetical genotype with regard to avirulence genes is given for certain races of this haploid fungus.

The final technique applied differed from that of Raa (1968b) used with apple and *V. inaequalis*. Tomato plant tissue was labelled with the radioactive isotope ^{32}P. This isotope was used because this strong β-emitter is an active ion in plant metabolism. We have also attempted to apply ^{36}Cl, but this isotope proved to be unsuitable.

High molecular weight substances from culture filtrates were utilized in the experiments. These were separated from the low molecular weight material with the use of gel filtration techniques. Gel filtration was chosen, partly because possible toxins were expected to be compounds with a high molecular weight and partly because of certain advantages this technique offers over the dialysis techniques.

Separation procedures have to be carried out at low temperature to preserve the high molecular weight products of the fungus. At low temperature separation by dialysis takes too much time during

which the substances may decay or may be affected by bacterial contaminants. Besides these facts hydrolytic enzymes *e.g.* cellulase can damage the dialysis membrane.
In our previous publications (Van Dijkman and Kaars Sijpesteijn, 1971, 1972; Kaars Sijpesteijn and Van Dijkman, 1971, 1972) we have already described model experiments in vitro explaining the hypersensitive reaction in tomatoes carrying the Cf 1, Cf 2, Cf 3 and Cf 4 genes for resistance. This chapter gives a full account of this work with the different genes for resistance.

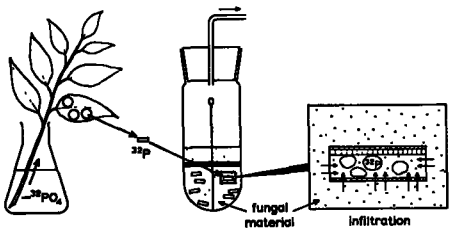
B Materials and Methods used in leakage experiments

1 *Plant and fungal material*
The plant varieties have been described in Chapter III. Healthy mature leaves (third and fourth leaf numbered from below) of 6 to 9 week old plants were used. Only in the experiments in which the influence of the age of the leaves of the variety V 121 was studied such leaves of 8 and 12 weeks old plants were used. The true genotypes of the plants are tabulated in Table I, Chapter II.
Careful inoculation experiments by Hubbeling (1968) have revealed that in the course of our work the genotype of the variety first being reported as Cf 1 Cf 2 cf 3 Cf 4 has to be changed into cf 1 Cf 2 cf 3 Cf 4. The first nomenclature for this variety was used in our previous publication (Van Dijkman and Kaars Sijpesteijn, 1971).
Leakage experiments were performed with leaf discs of the following tomato varieties: Moneymaker (cf), Leaf Mould Resister No. 1 (Cf 1), Vetomold (Cf 2), V 121 (Cf 3), Purdue 135 (Cf 4), V 473 (Cf 1 Cf 2) and Vagabond (Cf 2 Cf 4) and purified culture filtrates from the following races: $A_1A_2A_3A_4$, $a_1A_2A_3A_4$, $A_1a_2A_3A_4$, $A_1A_2A_3a_4$, $a_1a_2A_3A_4$, $a_1a_2a_3A_4$, $A_1a_2a_3a_4$ and $a_1a_2A_3a_4$.
The different physiological races used were grown in shake cultures in the protein-free casamino acids – glucose medium as described in Chapter III. The races used in this chapter are tabulated in Table I, Chapter II.

2 *Sephadex gel filtration of culture filtrates*
To separate low and high molecular weight material of the culture filtrate of *C. fulvum* Sephadex G-25 gel filtrations were performed. Shake cultures of *C. fulvum* were filtered through cotton wool or filter paper and applied to a column (3.8 x 91 cm) or a K 100/100 column (Pharmacia, Uppsala, Sweden) filled with Sephadex G-25 medium grade. The columns were fitted with a cooling jacket. The void volumes (V_0) of both columns were determined with the coloured high molecular weight polysaccharide Blue Dextran 2000. The total volume V_t was calculated from the volume of the gel bed of the columns. Gel filtrations were performed at 4 °C with glass-distilled water. The fractions were collected either by hand or with an L.K.B. 7000 Ultra Rac Fraction collector (L.K.B., Stockholm, Sweden), and freeze-dried (Cenco Virtis freeze-drier, Gardiner, New York, U.S.A.).
Fractions were stored in a deepfreezer until use.

3 *Labelling of tomato leaf*
To obtain ^{32}P-labelled tomato tissue, leaves were cut off and placed in flasks with water containing ^{32}P-orthophosphate, carrier-free (standard solution

Scheme 1 Experimental procedure for the leakage experiments.



1 mCi/ml). The leaves were illuminated with a 250W lamp and a slight current of air was passed over them to hasten uptake. The ^{32}P-orthophosphate solution had been taken up completely within 3 h. Total radioactivity taken up by the leaves and stems did not exceed 300 μCi. The radioactive isotope ^{36}Cl was used only once.

4 *Infiltration of tomato leaf discs*

After complete uptake of the isotope, discs were cut out from the intravenal areas by means of a specially sharpened 8-mm corkborer. Discs were then distributed at random over the infiltration vessels (50 in each) to obtain equal radioactivity in all flasks. Subsequently the discs were washed with a buffer solution (Tris-HCl, 10^{-2}M, pH 6) and finally placed in 5 ml of the same buffer solution containing the freeze-dried V_0-fraction (Figs. 1 and 2). Unless otherwise stated, the concentration of this solution was adjusted to that of the unpurified culture filtrate on basis of protein content for the fungal race in question. This means that the protein concentration varied from approx. 2–4% w/v.

The solution, containing the V_0-fraction, was prepared by dissolving the powder in an erlenmeyer flask placed in an ice bath under continuous magnetical stirring. The solution was freed from undissolved material by centrifuging for 10 min. at 10.000 g at 4 °C.

Leaves were slowly infiltrated with this solution of the V_0-fraction in vacuo for approx. 30 min. The procedure was accomplished in an ice-bath to avoid boiling, foaming and subsequent denaturation of the proteins in solution. Air was readmitted into the vacuumed flasks through a small capillary tube at a very slow rate to avoid cell damage from pressure shock. The whole procedure is illustrated in Scheme 1. Different buffers and solutions were tested in our leakage experiments to investigate which buffer causes the lowest background leakage of the tomato leaf cells following the pressure shock of the infiltration procedure. The following systems were tested: 0.01 M potassium phosphate (pH 6), 0.01 M Tris-HCl (pH 6), 0.35 M saccharose, 0.35 M NaCl, 0.35 M saccharose – 0.35 M NaCl and distilled water.

5 *Sampling and counting of ^{32}P leakage*

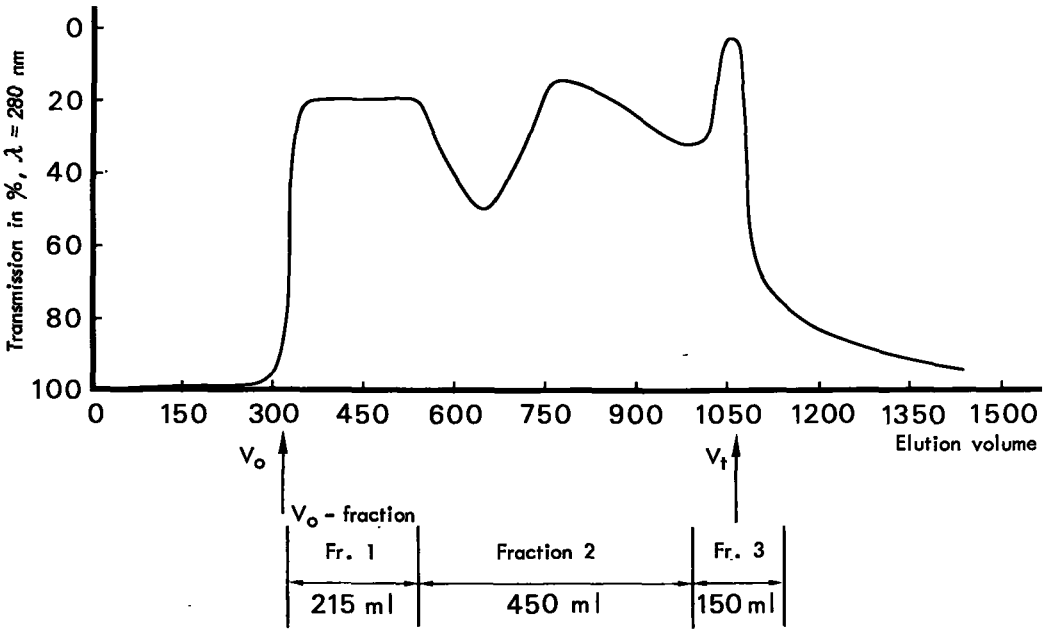
Phosphorus (^{32}P) was analysed in the liquid starting immediately after infiltration. Samples (0.1 ml) were removed at intervals, placed in scintillation flasks containing 15 ml water and counted in a liquid scintillation counter (Nuclear, Chicago). At the end of the leakage tests, the remaining radioactivity in the leaf was determined by ashing the dried leaf discs in a scintillation flask with pure oxygen (Micro-Mat, BF 5010, Berthold-Frieseke, Karlsruhe, B.R.D.) and dissolving the resulting ash in 0.35 M HCl. Phosphorus (^{32}P), a strong β-emittor, requires no additional scintillators for detection. The Cherenkov radiation was measured directly in water. No quenching was observed by the samples or chemicals used.

Only the countings underlying the results given in Fig. 5A and 5B, Fig. 6 and Fig. 7 were performed on a conventional end-window G-M counter tube. For that purpose 0.1-ml samples were dried on aluminium planchets and counted. The rest of the procedure was identical with the other experiments.

6 *Calcultations*

Total radioactivity of leaf disc material initially

Fig. 1 Characteristic elution pattern of Sephadex G-25 gel filtration of culture filtrate of *C. fulvum* race $A_1a_2A_3A_4$. Sample volume 225 ml.

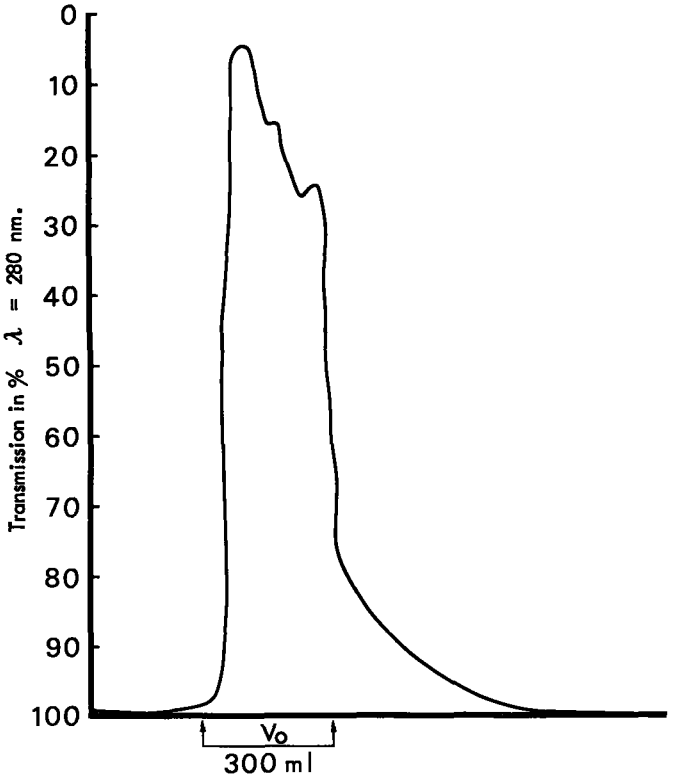


present was obtained from the sum of radioactivity of ash, liquid samples, and remaining liquid in the flasks. Radioactivity of liquid fractions represents leakage of phosphorus from plant cells. The leakage of phosphorus from discs was expressed as percentage of maximum leakable ^{32}P. The maximum amount of ^{32}P leakable from discs follows infiltration by 2% (v/v) chloroform in buffer (Van Dijkman and Kaars Sijpesteijn, 1971). Chloroform infiltration included in the experiments caused leakage of approximately 75% of leaf phosphorus. The leakage into chloroform solutions was completed within 30 minutes after start of infiltration.
In the calculations the decay factor for radioactivity of ^{32}P was used where necessary. For large-scale experiments calculations were performed on the Electrologica X-8 computer of the Mathematical Institute of the State University of Utrecht.

C Results

1 *High molecular weight material from culture filtrates of races of C. fulvum*
In Fig. 1 the absorption at 280 nm of the elution of a culture filtrate of *C. fulvum* from a Sephadex G-25 column is given. The sample volume was 225 ml. In general three fractions were collected. Fraction 1 is the void-volume (V_0) fraction containing molecules with a molecular weight above 1000. Fraction 2 consists of the compounds which can be separated by their molecular weight on a Sephadex G-25 column. Fraction 3 is the total volume (V_t) fraction, containing the small molecules which cannot be separated by gel filtration over Sephadex G-25. In our experiments, only the V_0-fractions were

Fig. 2 Elution pattern of Sephadex G-25 gel filtration on a K 100/100 column of the combined V_0 -fractions of *C. fulvum* race $A_1a_2A_3A_4$.



collected because the specific activity we were looking for appeared to be present in these particular fractions (*cf.* Section 3).
Absorption measurements at 280 nm of effluents of culture filtrates of the other races of *C. fulvum* separated on Sephadex G-25 gave similar absorption patterns. The level of the absorption varied with the length of the incubation period.
The fractions were collected in the fraction collector and subsequently freeze-dried. The resulting greyish powder was stored at –30 °C until use.
The void volume fractions of different batches of each physiological race of *C. fulvum* were combined and purified once more over Sephadex G-25 to obtain a standard preparation for use in the experiments to be described. Fig. 2 gives the elution pattern (λ = 280 nm) of such a purified preparation as recorded by the Uvicord spectrophotometer.
Calculated on the applied quantity of the preparation it was obvious that this fraction did not contain any molecules with a molecular weight smaller than 1000, which absorbed at 280 nm. A greyish powder was obtained and stored in the deepfreezer. This second gel filtration over Sephadex G-25 of the fungal material yielded often a cleaner preparation after freeze-drying with a colour tending towards white.
Protein concentration was determined with the Folin-phenol method. Table I gives the content of protein present in the combined freeze-dried V_0-fraction of different races of *C. fulvum*. This ranges for the different races of *C. fulvum* from 27 to 60%, w/w.
To measure the concentration of protein in the V_0-fraction the spectroscopical method was unsuitable because of a magenta pigment cladofulvine, a 1.2.8-trihydroxy-7-methylanthraquinone (Thomson, 1971), produced by most *C. fulvum* races. This compound is adhered to substances with a molecular weight above 1000, and thus is present in the V_0-fractions of a gel filtration over Sephadex G-25. Calculations on the basis of the A_{280} therefore gave inconsistent results. The total amount of protein varied with the length of time of incubation of the cultures.
Different nutritional circumstances did not greatly influence the protein content of the V_0-fraction when yields of mycelial growth were standardized. The growth period required for optimum production of active components was tentatively based on the maximum amount of total protein present in the V_0-fraction of the culture filtrates. Fig. 3 represents the total protein present in the V_0-fraction of the culture filtrate of race $a_1A_2A_3A_4$ after 14, 27, 38, 40, 46 and 50 days of incubation. This experience led us to incubate the shake cultures for the production of high molecular weight material from *C. fulvum* races for three to four weeks.

2 *First attempts to study the effect of fractions of C. fulvum culture filtrates on tomato plant tissue*
Several model experiments were designed to study the effect of excretion products of the various races of *C. fulvum* on tissues of the different host varieties. Various procedures were tried to investigate this interaction.
Analogous to the wilting experiments of Raa (1968b) described before, we tried wilting experiments with tomato and fractions of the culture filtrates of compatible and incompatible combinations of *C. fulvum*. Tomato cuttings were placed on solutions containing the dialyzed culture filtrates of compatible and incompatible races of *C. fulvum* or on

Fig. 3 The relation between incubation period at 24° C and total protein content of the freeze-dried V_o-fraction of culture filtrates of *C. fulvum* race $a_1A_2A_3A_4$.

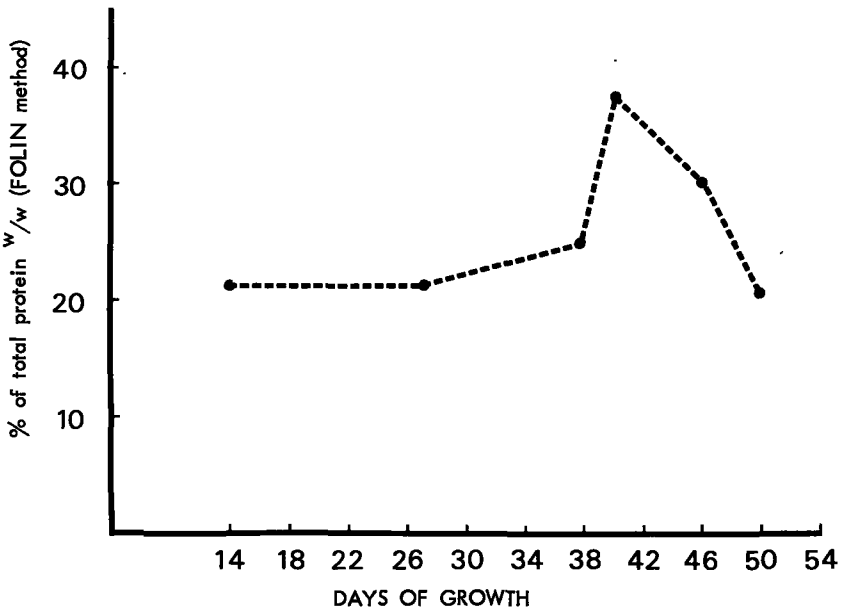


Table I Total protein content of the combined freeze-dried Sephadex V_o-fraction of various physiological races of *C. fulvum* (Folin-phenol method).
The shake cultures were incubated for 3–4 weeks at 24° C.

Physiological race of *C. fulvum*	Percentage of total protein in dry weight
$A_1A_2A_3A_4$	27
$a_1A_2A_3A_4$	41
$A_1a_2A_3A_4$	45
$A_1A_2A_3a_4$	38.5
$A_1a_2a_3a_4$	60
$a_1a_2A_3a_4$	56

Fig. 4A and B Leakage of ^{32}P from leaf discs of tomato variety Moneymaker (Fig. 4A) and Leaf Mould Resister No. 1 (Fig. 4B) infiltrated with various buffers and solutions: 0.01 M phosphate buffer (pH 6); 0.01 M Tris-HCl buffer (pH 6); 0.35 M saccharose; 0.35 M NaCl; 0.35 M saccharose + 0.35 M NaCl; distilled water.

solutions containing the dissolved Sephadex G-25 V_0-fraction; in all cases wilting was observed within a relatively short time. These experiments were unsuccessful probably because tomato cuttings are in general very liable to wilting. Tomato plant cuttings in water and in the buffer control also wilted in a short time after the initiation of the experiments. No differences were observed between compatible and incompatible combinations; they all wilted. Moreover, the relatively high viscosity of the tested solution, caused by the presence of fungal polysaccharides, presumably plugged the vessels.

Another technique used in similar studies (Pellizzari *et al.*, 1970) was to measure the leakage of ions out of the tomato tissue. The procedure was practically identical with that of the radioactive experiment to be described below. In this trial we measured the increase of conductivity with a modified Wheatstone bridge (Phyloscope Philips GM 1044). Unfortunately no difference was found between infiltrations of leaf discs with fractions from compatible and incompatible combinations of tomato and *C. fulvum*. The background conductivity after the infiltration procedure was too high to measure any significant differences. Another disadvantage for the application of this technique is that the solubility of the V_0-fractions of the culture filtrates is rather poor in water with a low conductivity. We used for these experiments de-ionized, double glass-distilled water. Expecting that high molecular weight material from the fungus, possibly proteins, should be responsible for the anticipated differences between compatible and incompatible combinations, a low ionic strength is disadvantageous for the solubility of such compounds. Leakage of characteristic constituent of tomato plants, like tomatine, might also provide a possibility to measure differences in leakage of compatible and incompatible races of *C. fulvum*. Tomatine is present in leaf cells of tomato in relatively large amounts; *i.e.* approx. 0.5–1% on a dry weight basis in commercial tomato varieties. This steroid-alkaloid glycoside can be detected in small amounts (10 μg) on silica thin layer plates after spraying with a 20% ethanolic solution of phosphormolybdic acid and subsequently followed by heating until the spots appear. Details on this compound are given in Chapter V. The results with this technique were rather poor. In a few cases the incompatible combinations did leak more than the compatible ones or the buffer control infiltration, but the differences were small. Moreover, a good quantitative test for tomatine does not yet exist and quantitative evaluation of the spots on the thin layer plates is quite impossible.

The experiments with labelled tissue were more successful and are described in the following section.

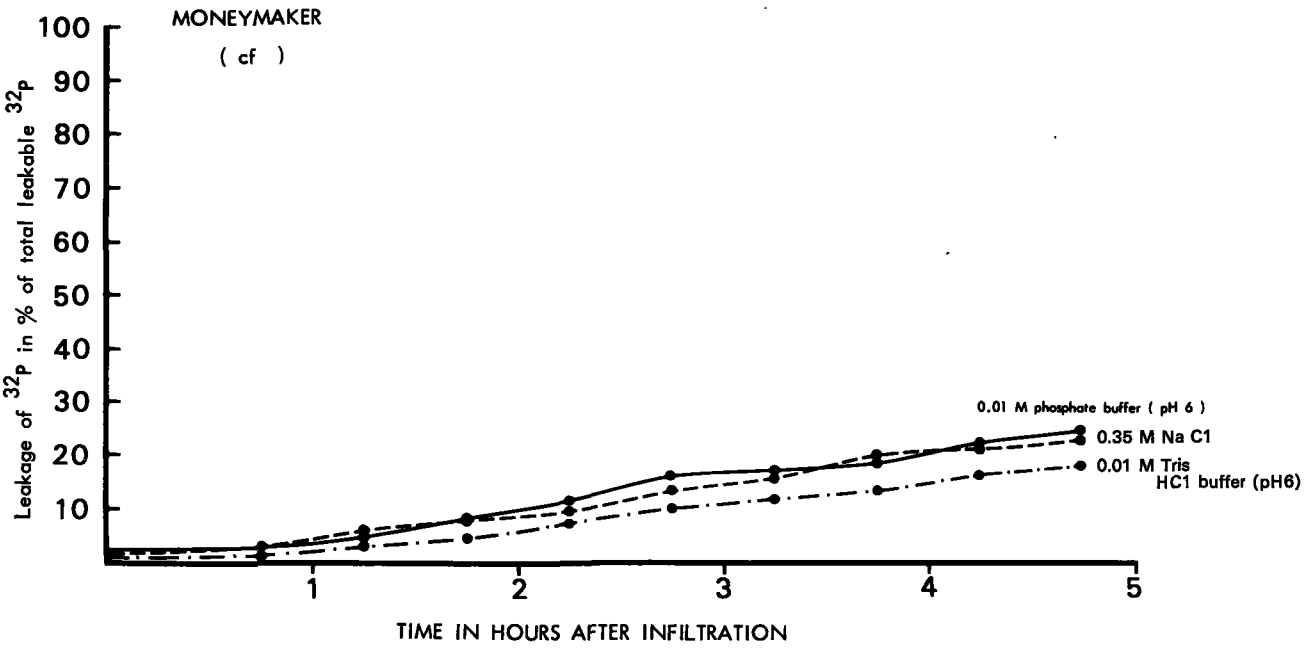
3 *Leakage of ^{32}P from labelled tomato tissue infiltrated with the V_0-fraction of culture filtrates of C. fulvum*

Since the V_0-fraction dissolves badly in water, it was necessary to find a proper medium to dissolve the preparation. Moreover, this medium should have a minimal toxicity for the plant cells.

Fig. 4A and 4B illustrate the results of leakage experiments with different buffers and other solutions. Evidently, the Tris-HCl buffer was most suitable for our leakage experiments.

In Table VII the combinations tested are given as well as those not described here. The figures presented below give the graphical reproduction of the results of the different leakage experiments.

For the leakage experiments, the tomato varieties Leaf Mould Resister No. 1 and Vetomold were used


MONEYMAKER
(cf)
Leakage of ^{32}P in % of total leakable ^{32}P
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
TIME IN HOURS AFTER INFILTRATION
0.01 M phosphate buffer (pH 6)
0.35 M Na C1
0.01 M Tris HC1 buffer (pH6)

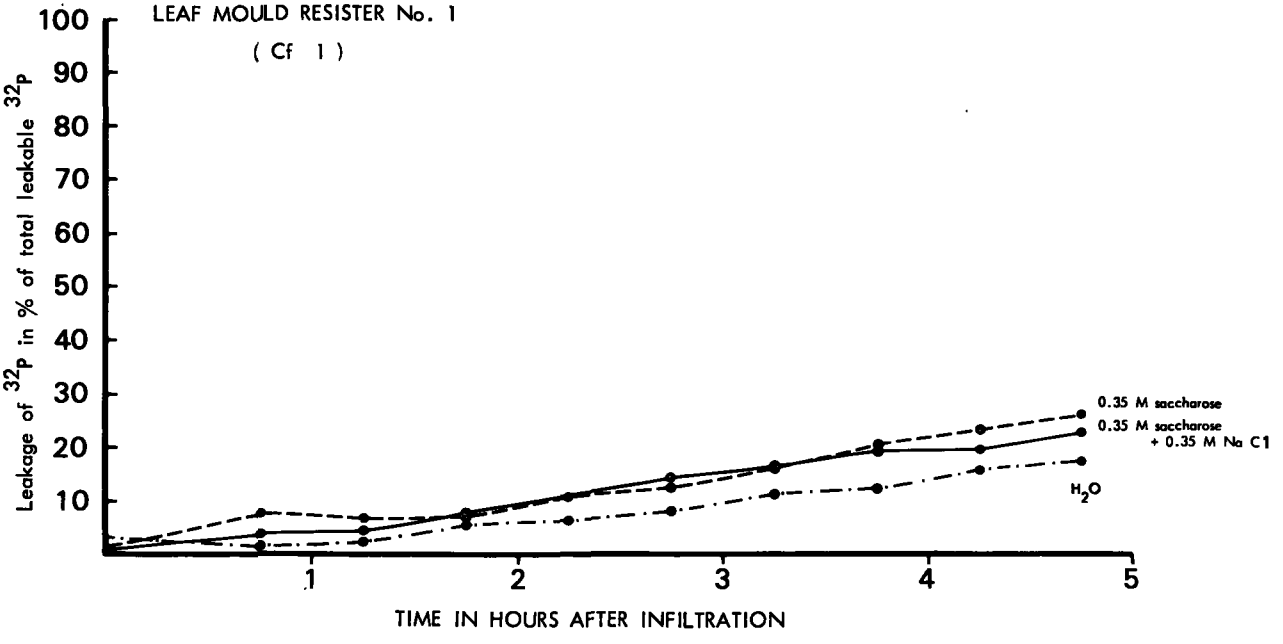
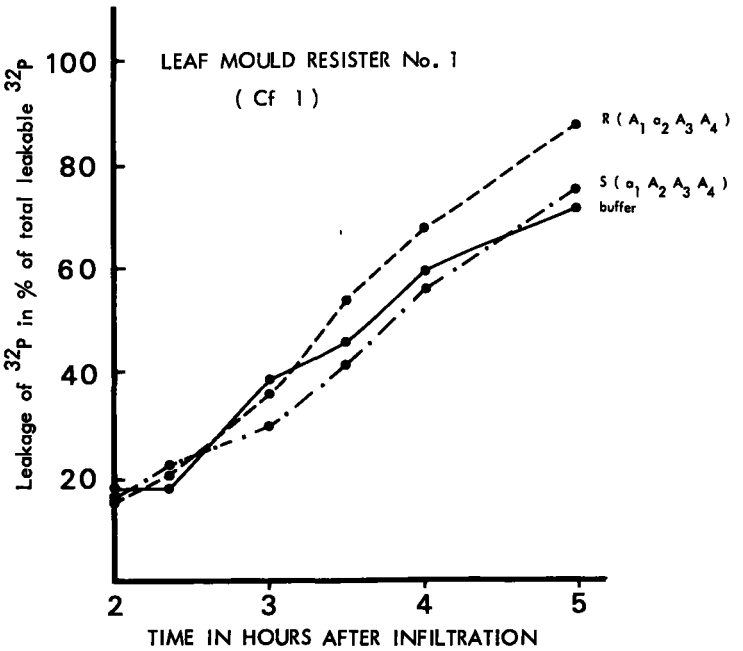

LEAF MOULD RESISTER No. 1
(Cf 1)
Leakage of ^{32}P in % of total leakable ^{32}P
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
TIME IN HOURS AFTER INFILTRATION
0.35 M saccharose
0.35 M saccharose + 0.35 M Na C1
H_2O

Fig. 5A and B Leakage of ^{32}P from tomato leaf discs infiltrated with V_o-fraction of culture filtrate of two races of *C. fulvum*.
A: Tomato variety Leaf Mould Resister Nr. 1 (Cf 1);
B: Tomato variety Vetomold (Cf 2);
R: Incompatible combination;
S: Compatible combination.



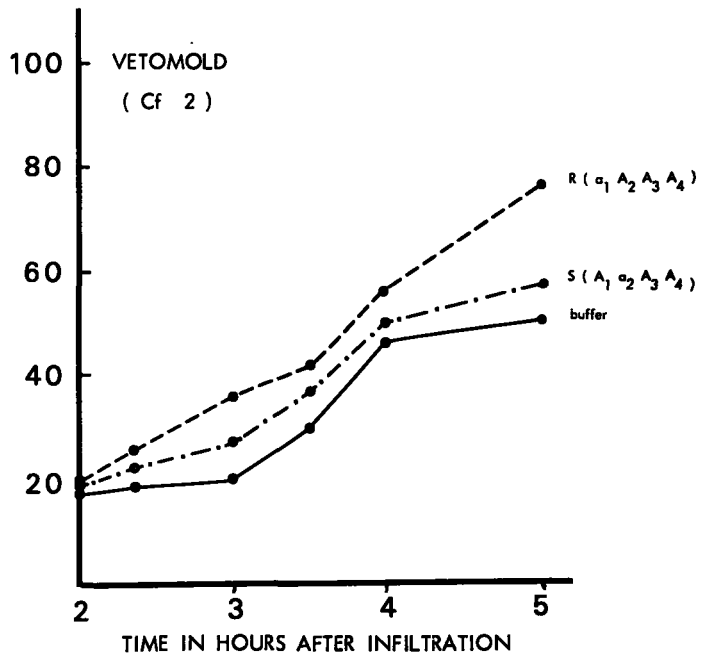
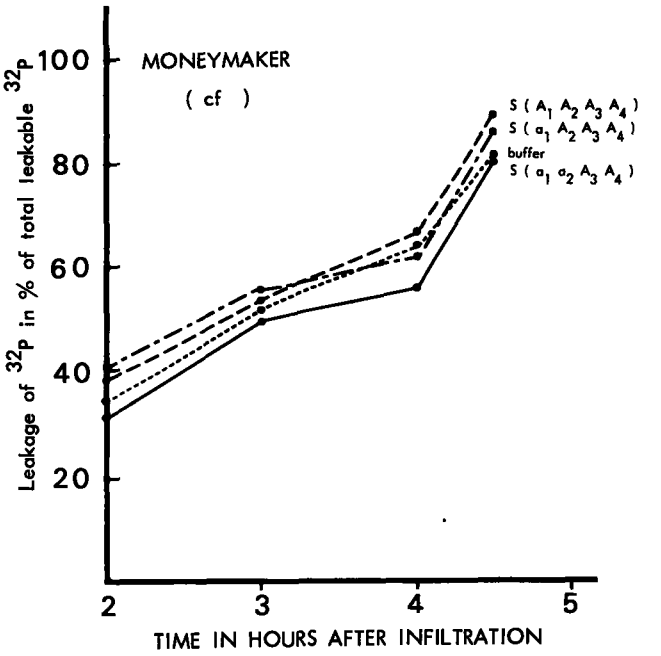


Table II Host relation of the varieties Leaf Mould Resister No. 1 and Vetomold and two fungal races.

		C. fulvum	
		$A_1a_2A_3A_4$	$a_1A_2A_3A_4$
Leaf Mould Resister No. 1	Cf 1	R	S
Vetomold	Cf 2	S	R

Fig. 6 Leakage of ^{32}P from leaf discs of tomato variety Moneymaker (cf), infiltrated with the V_o-fraction of culture filtrate of *C. fulvum* races.
R: Incompatible combination;
S: Compatible combination.



in combination with the races $A_1a_2A_3A_4$ and $a_1A_2A_3A_4$ of *C. fulvum*. The relationship between these races and the two tomato varieties are given in Table II.
The results of the leakage experiments with these combinations are given in Fig. 5A and 5B. Leakage of ^{32}P after different time intervals is illustrated.
The extent of leakage of labelled compounds from the discs is given in relation to the time elapsed after infiltration of the excretion products of the fungi.
Leaf Mould Resister Nr. 1 (Cf 1) is susceptible to the race $a_1A_2A_3A_4$ and infiltration with products of this race does not cause a leakage which exceeds that caused by infiltration of the buffer solution alone. The same fungal products, however, cause strong leakage in excess of the buffer control after infiltration into Vetomold (Cf 2), which is resistant to this race $a_1A_2A_3A_4$. Quite the opposite situation was found for the race $A_1a_2A_3A_4$ which is able to attack Vetomold (Cf 2) but not Leaf Mould Resister Nr. 1 (Cf 1): we found strong leakage of Leaf Mould Resister Nr. 1, but not of Vetomold. These results are strongly in favour of our hypothesis of selectively toxic products being excreted by the pathogen.
Fig. 6 illustrates experiments with tissue from the variety Moneymaker, which is recessive to all resistance genes and susceptible to all races of the fungus. After infiltration of leaf discs of the variety Moneymaker with V_0-fractions of three different races of *C. fulvum* $A_1A_2A_3A_4$, $a_1A_2A_3A_4$ and $a_1a_2A_3A_4$ no excess of leakage to the buffer control occurred. Presumably no receptor sites for toxins causing membrane damage are present in the cells of this variety.
Similar correlations as with Leaf Mould Resister and Vetomold are observed with the tomato variety V 473.

Fig. 7 Leakage of ^{32}P from leaf discs of tomato variety V 473 (Cf 1 Cf 2), infiltrated with the V_o-fraction of culture filtrate of *C. fulvum* races.
R: Resistant combination;
S: Susceptible combination.

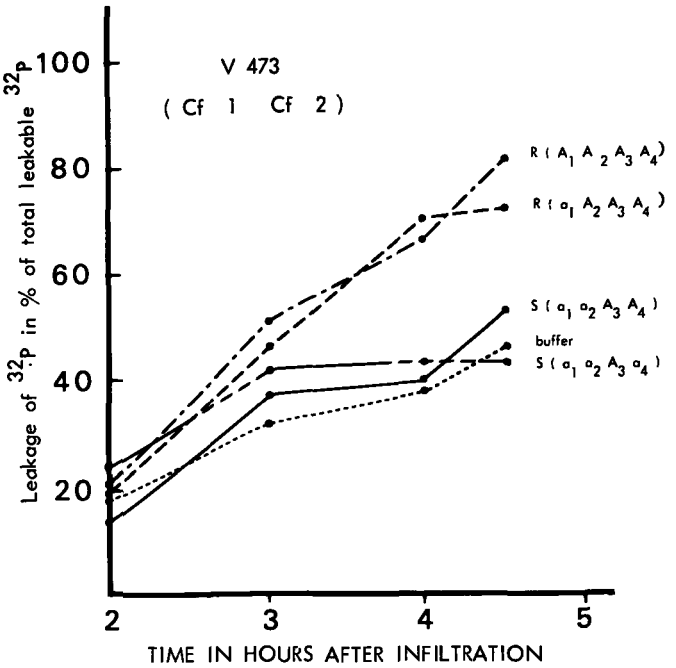
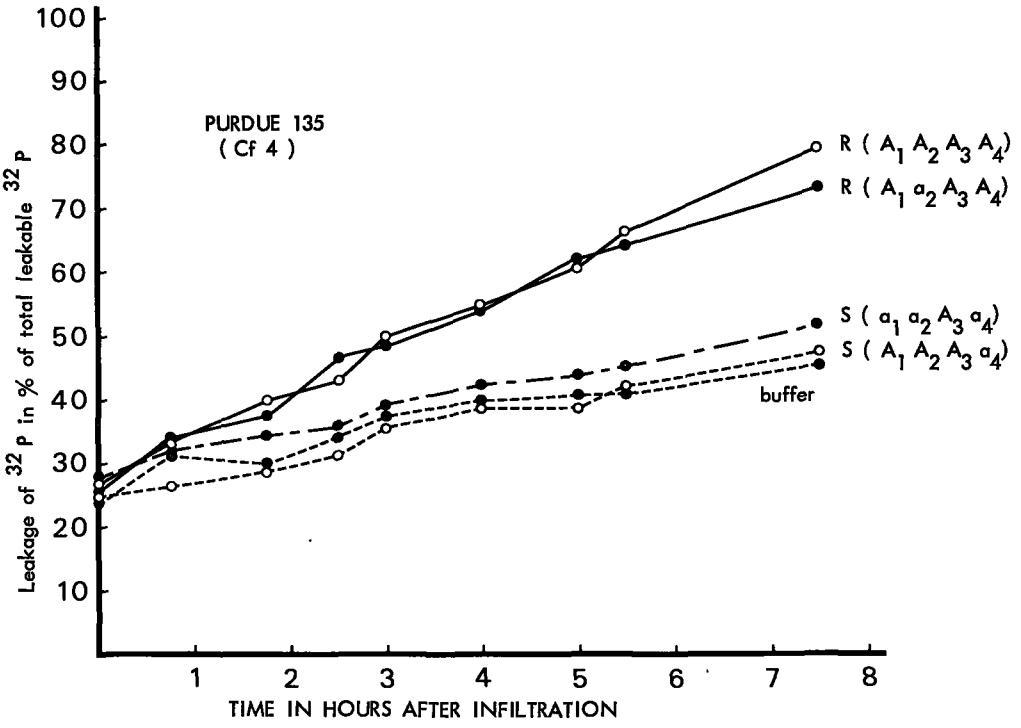


Table III Host relation of the variety V 473 (Cf 1 Cf 2) and four fungal races.

Tomato variety	Physiological races of *C. fulvum*			
	$A_1A_2A_3A_4$	$a_1A_2A_3A_4$	$a_1a_2A_3A_4$	$a_1a_2A_3a_4$
V 473 Cf 1 Cf 2	R	R	S	S

The variety with the genes Cf 1 as well as Cf 2, was treated with the V_0-fraction from the races $A_1A_2A_3A_4$, $a_1A_2A_3A_4$, $a_1a_2A_3A_4$ and $a_1a_2A_3a_4$. The host-parasite relation between these fungi and the plant is given in Table III. The results of this leakage experiment is given in Fig. 7.
Fig. 8 shows a leakage experiment with leaf discs from the tomato variety Purdue 135 carrying the dominant allele Cf 4. The races $A_1A_2A_3a_4$ and $a_1a_2A_3a_4$ attack this tomato variety and their culture filtrates appear unable to cause after infiltration leakage of the cells which is in excess of that by the buffer control. In contrast preparations of the races $A_1A_2A_3A_4$ and $A_1a_2A_3A_4$ which are incompatible with this tomato do cause extra leakage over the buffer control as well as over the compatible combinations. These results are also in line with the leakage experiments of Leaf Mould Resister, Vetomold and V 473.
Finally leakage experiments were carried out with the combinations described in Table IV (Fig. 9A, 9B and 9C). These combinations were chosen because the host relation of the tomato varieties Vetomold (Cf 2), and Purdue 135 (Cf 4), towards the two physiological races is opposite.
Moreover, the genotype of the avirulence alleles of these two fungal races, $A_1A_2A_3a_4$ and $a_1a_2a_3A_4$, is complementary. For further comparison the tomato Vagabond (Cf 2 Cf 4) was included in this experiment. Similar to Vetomold the resistance of Vagabond to the race $A_1A_2A_3a_4$ is due to the presence of Cf 2 whereas resistance of Vagabond to the race $a_1a_2a_3A_4$ is ascribed to Cf 4, like in Purdue. The results show again that leakage of incompatible combinations exceeds that of compatible combinations and the buffer control.

Fig. 8 Leakage of ^{32}P from tomato leaf discs of the variety Purdue 135 (Cf 4) infiltrated with the Sephadex G-25 V_o-fraction culture filtrates of four races of *C. fulvum*.



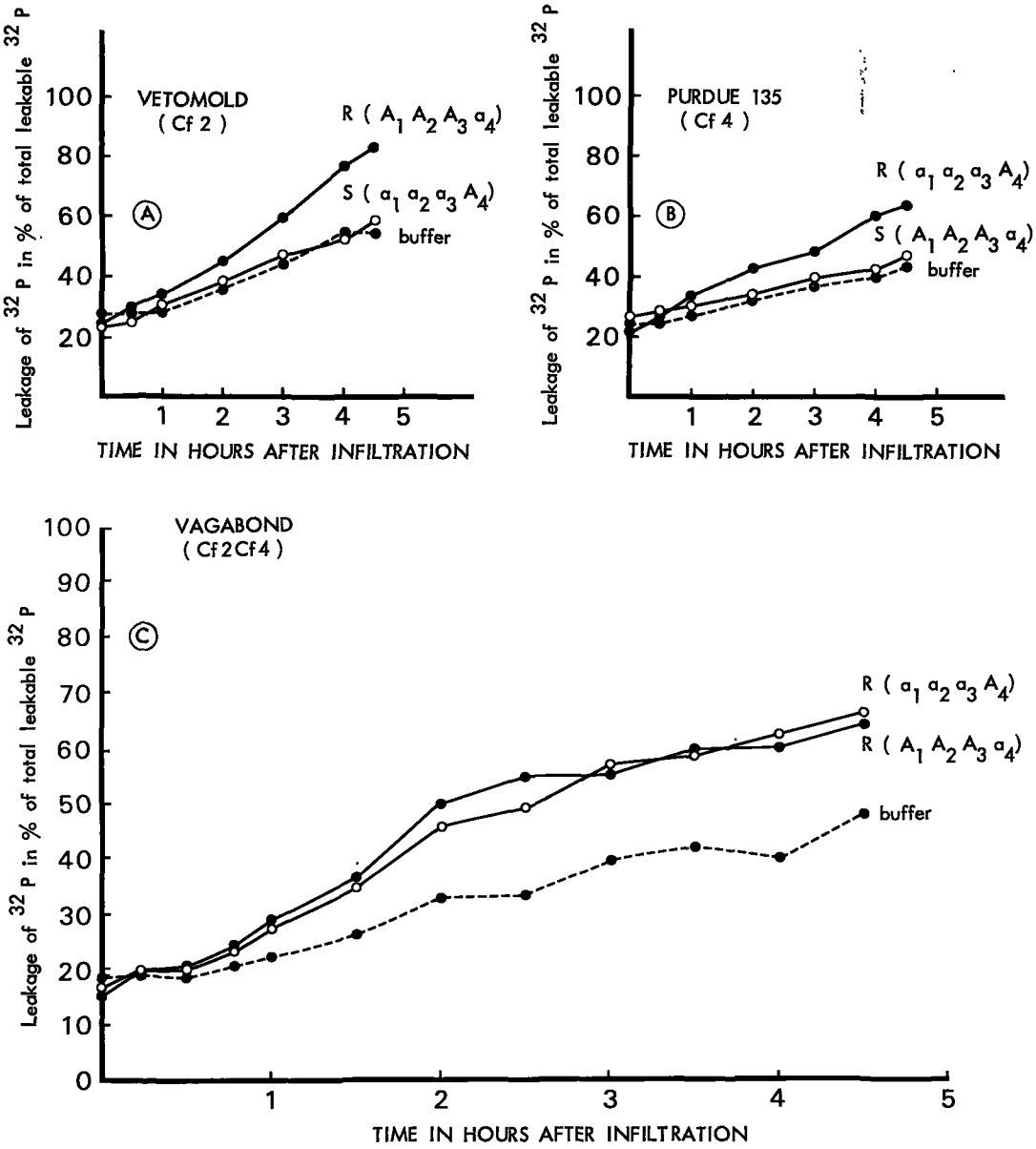
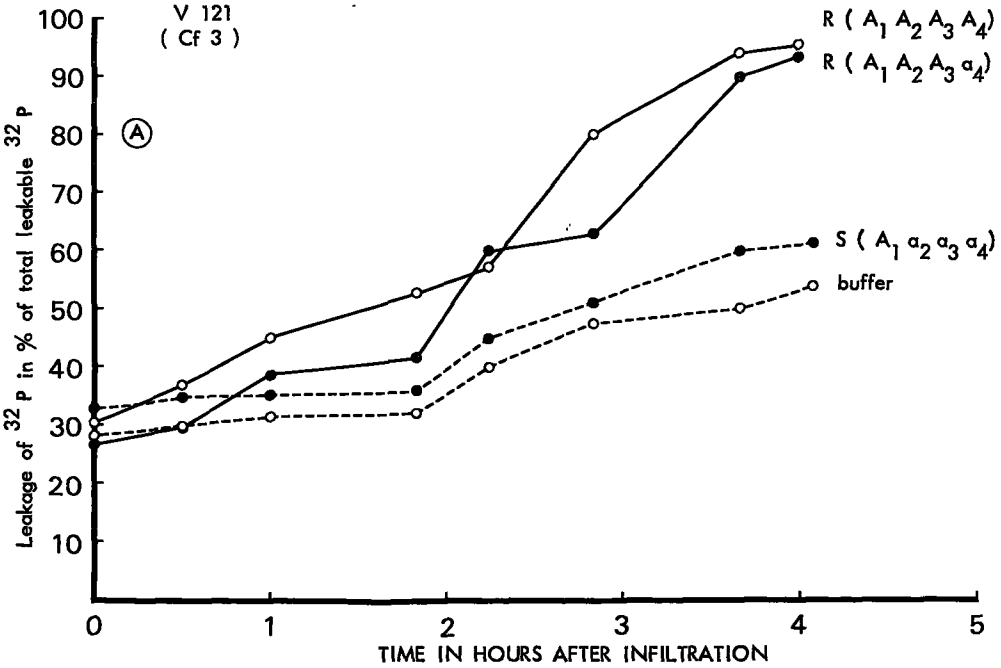

VETOMOLD
(Cf 2)
R (A_1 A_2 A_3 a_4)
S (a_1 a_2 a_3 A_4)
buffer
Leakage of ^{32}P in % of total leakable ^{32}P
TIME IN HOURS AFTER INFILTRATION
PURDUE 135
(Cf 4)
R (a_1 a_2 a_3 A_4)
S (A_1 A_2 A_3 a_4)
buffer
Leakage of ^{32}P in % of total leakable ^{32}P
TIME IN HOURS AFTER INFILTRATION
VAGABOND
(Cf2Cf4)
R (a_1 a_2 a_3 A_4)
R (A_1 A_2 A_3 a_4)
buffer
Leakage of ^{32}P in % of total leakable ^{32}P
TIME IN HOURS AFTER INFILTRATION

Fig. 9 Leakage of ^{32}P from leaf discs of the tomato varieties Vetomold (A), Purdue 135 (B) and Vagabond (C), infiltrated with the Sephadex G-25 V_o-fraction of culture filtrates of two physiological races of *C. fulvum*. In Fig. 9C the abscis and the ordinate differ from those in Fig. 9A and 9B.

Leakage experiments were also performed with leaf discs of the tomato variety V 121, with the Cf 3 gene. The discs were infiltrated with the freeze-dried V_0-volume fractions of a Sephadex G-25 gel filtration of the culture filtrates from the compatible race of $A_1a_2a_3a_4$ of *C. fulvum* and from the two incompatible races $A_1A_2A_3A_4$ and $A_1A_2A_3a_4$. The results in Fig. 10A show also quite convincingly that leakage in the incompatible combination far exceeds that in the compatible combination. In the latter case the extent of leakage is more or less the same as that caused by the buffer control. The results of this experiment involving the Cf 3 allele for resistance, are thus very much in line with our earlier results involving the alleles Cf 1, Cf 2, Cf 4 and the combinations of these alleles Cf 1 Cf 2 and Cf 2 Cf 4. After a month's interval this experiment was repeated using third and fourth leaves of the same set of plants (Fig. 10B). Results are quite comparable to those given in Fig. 10A. Yet there is a remarkable difference. The whole level of leakage including that by the control buffer appears to be much lower. This may mean that younger leaves are most sensitive than older leaves to the infiltration treatment as shown by a higher non-specific leakage of the membranes. The times of sampling in these leakage experiments cannot be extended too long. In most experiments 6 hours is the maximum. Bacterial contamination disturbs the experiments and decreases the differences between the leakage of the compatible and the incompatible combinations. After approx. 20 h the differences have completely vanished.
Not all experiments carried out have been described in this chapter. Results consistently were in confirmation with our hypothesis. All combinations tried have been assembled in Table VII.

Table IV Host relation of the three tomato varieties and the two fungal races used in the experiments given in Fig. 9A, 9B and 9C.

Tomato variety		Physiological races of *C. fulvum* $A_1A_2A_3a_4$	$a_1a_2a_3A_4$
Vetomold	Cf 2	R	S
Purdue 135	Cf 4	S	R
Vagabond	Cf 2 Cf 4	R	R


V 121
(Cf 3)
A
R (A1 A2 A3 A4)
R (A1 A2 A3 a4)
S (A1 a2 a3 a4)
buffer
Leakage of 32 P in % of total leakable 32 P
TIME IN HOURS AFTER INFILTRATION

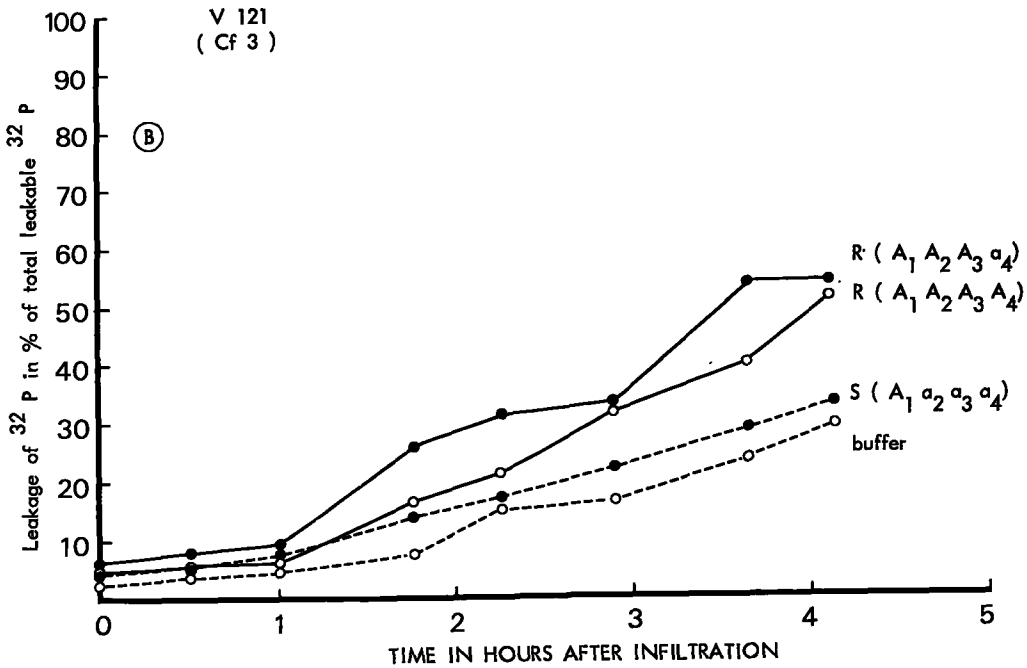

V 121
(Cf 3)
B
R (A1 A2 A3 a4)
R (A1 A2 A3 A4)
S (A1 a2 a3 a4)
buffer
Leakage of 32 P in % of total leakable 32 P
TIME IN HOURS AFTER INFILTRATION

Fig. 10 Leakage of ^{32}P from tomato leaf discs taken from the third and fourth leaf of the variety V 121 (Cf 3) infiltrated with the Sephadex G-25 V_0-fraction of the culture filtrates of three races of *C. fulvum*.
A: Discs taken from young leaves (6–9 weeks);
B: Discs taken from old leaves (8–12 weeks).

4 *Required concentration of the Sephadex G-25 V_0-fraction*
To investigate the effect of dilution of the V_0-fraction on leakage the following experiment was carried out: leaf discs of the variety Purdue 135 (Cf 4 allele for resistance) were infiltrated either with a V_0-fraction of Sephadex G-25 gel filtration of the incompatible race $A_1A_2A_3A_4$ or with the V_0-fraction of the race $A_1A_2A_3a_4$, which race can attack Purdue 135. Four concentrations of the fractions were used: the usual concentration i.e. containing the same protein content as the original culture filtrate, a 1:2, a 1:4 or a 1:16 dilution thereof. Obviously, in incompatible combinations the dilution of the preparation leads to a gradual decline of leakage of ^{32}P-containing compounds from the leaf discs (Fig. 11). However, with compatible combinations there was no effect of dilution of fractions.
Evidently, to observe leakage in the model experiments the concentration of the active fraction cannot be greatly diminished beyond that of the original culture filtrate, at least in the combination Purdue 135 (Cf 4) and physiological race $A_1A_2A_3A_4$ of *C. fulvum*.

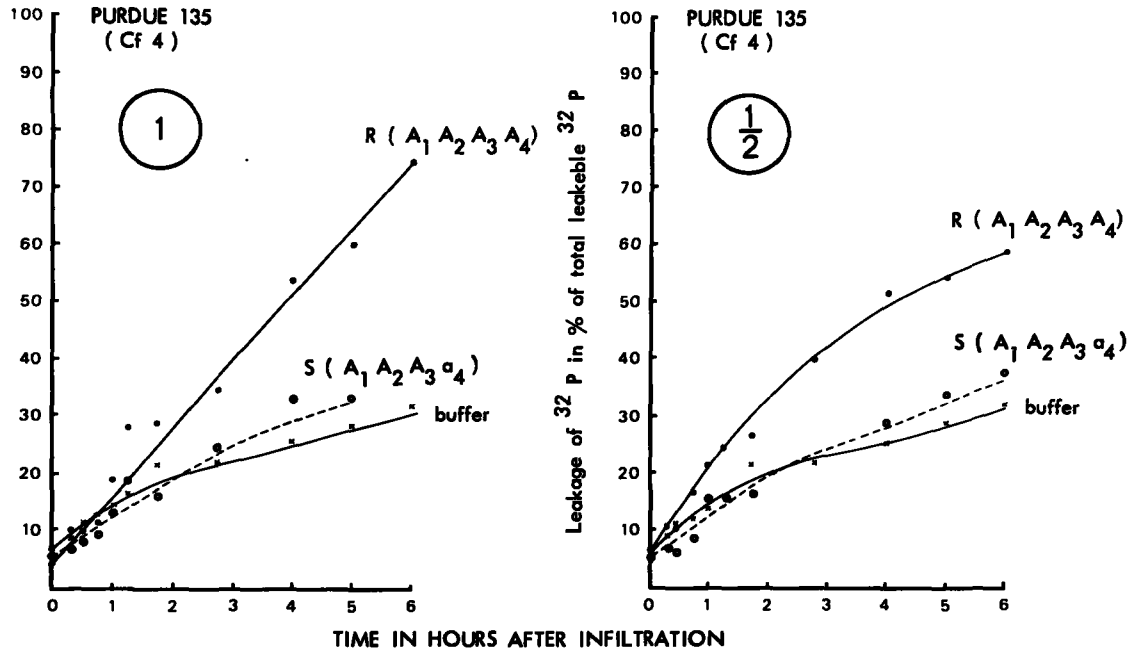
5 *The activity of other fractions besides the V_0-fraction of culture filtrates of C. fulvum*
In Fig. 1 the absorbance of the total effluent of the gel filtration over Sephadex G-25 from a culture filtrate of *C. fulvum* $A_1a_2A_3A_4$ was recorded. The total volume is divided into three parts: Fraction 1 is the V_0-fraction containing molecules with a molecular weight $\geqslant$ 1000, calculated on the basis of the separation of globular protein over Sephadex G-25. This fraction proved active in our leakage experiments. With the varieties Moneymaker, Leaf Mould Resister Nr. 1 and Vetomold, we also tested the activity of Fraction 2, with molecules in the range 100 to 1000 molecular weight and Fraction 3 which contains molecules with a molecular weight $\leqslant$ 100. Only compounds in the V_0-fraction bring about leakage in the incompatible combination which exceeds that of the control and the compatible combinations. Thus we must conclude that the active compounds have a molecular weight $\geqslant$ 1000.

6 *Leakage experiments with ^{36}Cl*
In leakage experiments with ^{36}Cl instead of ^{32}P no leakage exceeding that of the buffer control was observed in incompatible combinations of Leaf Mould Resister No. 1 (Cf 1) and Vetomold (Cf 2) and *C. fulvum* $A_1a_2A_3A_4$ and $a_1A_2A_3A_4$ V_0-fractions. Calculations of the data show that the radioactivity of the infiltrated liquid reached a constant level in all cases very soon after the infiltration procedure was stopped.

7 *Statistical treatment of data of the leakage experiments*
The graphs of the leakage experiments of section 2–5 can be approximated by straight lines. The equation of these lines is:

$$l = a \times t + b$$

where l is % of leakage. The slope, a, and intercept, b, were calculated by the method of least squares. t is the time in minutes.
The standard deviation (s) of the slopes and the correlation coefficients (r) were estimated (*cf.* Kelly, 1963).
Fig. 8 and 9B represent the leakage experiments with the variety Purdue 135 (Cf 4) and the races of


PURDUE 135
(Cf 4)
1
R (A1 A2 A3 A4)
S (A1 A2 A3 a4)
buffer
PURDUE 135
(Cf 4)
1/2
R (A1 A2 A3 A4)
S (A1 A2 A3 a4)
buffer
Leakage of 32 P in % of total leakable 32 P
TIME IN HOURS AFTER INFILTRATION

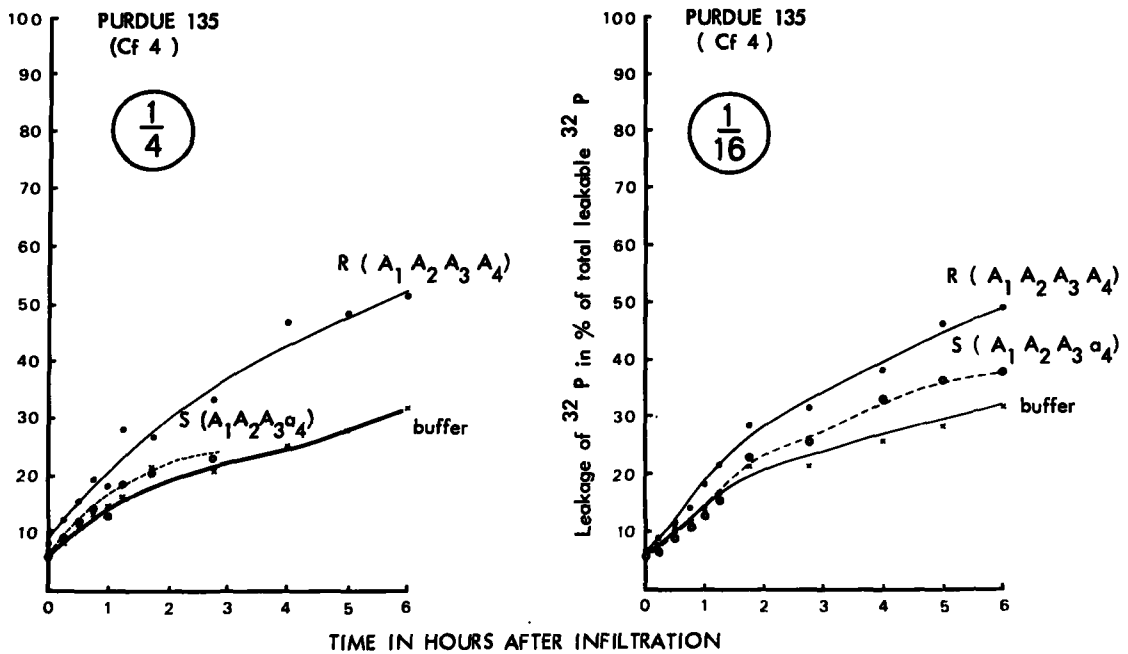

PURDUE 135
(Cf 4)
1/4
R (A1 A2 A3 A4)
S (A1A2A3a4)
buffer
PURDUE 135
(Cf 4)
1/16
R (A1 A2 A3 A4)
S (A1 A2 A3 a4)
buffer
Leakage of 32 P in % of total leakable 32 P
TIME IN HOURS AFTER INFILTRATION

Fig. 11 Dilution of the Sephadex G-25 V_o-fraction in a leakage experiment with tomato variety Purdue 135 (Cf 4) and the high molecular weight material of *C. fulvum* race $A_1A_2A_3A_4$ and $A_1A_2A_3a_4$. The numbers in circles indicate the dilution ratio.

C. fulvum $A_1A_2A_3a_4$, $a_1a_2A_3a_4$, $A_1a_2A_3A_4$ and $a_1a_2a_3A_4$. The calculations for these combinations are tabulated in Table V.
Fig. 10A and 10B show the results of leakage experiments with the tomato variety V 121 (Cf 3) and with the compatible race $A_1a_2a_3a_4$ and the incompatible races $A_1A_2A_3A_4$ and $A_1A_2A_3a_4$. The calculations for these combinations are arranged in Table V which represents the results of experiments with young and old leaves from the variety V 121 (Cf 3) (*cf.* section C 3).
These calculations (Table V and VI) reveal 1) that r is larger than 0.95 in all cases. The series of points can be fitted to straight lines; 2) that the differences between the slopes of the lines for the compatible and the incompatible combinations are significant.
Comparing the results for young and old leaves given in Table VI one observes a striking difference in the intercept. The value of the intercept corresponds with the initial leakage caused by the infiltration procedure. Obviously, young leaves are most sensitive to this procedure than older ones. Also the slopes are lower in the incompatible combinations where older leaves are involved. Apparently leakage in older leaves proceeds at a lower speed, although the ratio aR/aS for both young and old leaves lies around 2; $aR/a_{\text{buffer}} \simeq 2$ and $aS/a_{\text{buffer}} \simeq 1$.

D Discussion

The results summarized in Table VII show that there is a strong correlation in the various incompatible combinations investigated between the occurrence of the hypersensitive reaction in experiments in vivo and of cellular leakage in model experiments involving infiltration by fungal products. These observations strongly support the hypothesis presented in Chapter II. Part of this work has already been given in our previous publications (Van Dijkman and Kaars Sijpesteijn, 1971, 1972; Kaars Sijpesteijn and Van Dijkman, 1971, 1972).
The formation of these specific fungal products is supposed to be controlled by specific avirulence alleles of the fungus. The dominant Cf alleles of the genes for resistance may be responsible for the presence of specific receptors in the plant cell membranes which in combination with specific compounds of the fungus lead in model experiments to leaking of the cells, and in vivo to a hypersensitive reaction. Results indicate that the hypothesis forwarded applies to the four known resistance genes, Cf 1, Cf 2, Cf 3 and Cf 4 as well as to the hypothetical avirulence genes.
The statistical calculations in this chapter indicate that the differences found in the leakage experiments between compatible and incompatible combinations are significant.
The mechanism of leakage is not yet understood. Further experiments on molecular and electron-microscopical level are required to explain the reactions taking place. It may be assumed that the plasma membrane is involved.
The active compounds may be proteins or polysaccharides. Their behaviour in Sephadex G-25 filtration and their elution from the column with the V_0-fraction suggest molecular weights of least 1000.
The protein concentration of the infiltration solutions used in our experiments is rather high (2–4% w/v) and 27–60% of the dry weight of the preparations consists of proteins. The remaining dry weight possibly represents polysaccharides. Presumably,

Table V Statistical calculation of the results given in Fig. 8 and 9B. Tomato variety Purdue 135 (Cf 4).

	Physiological races of *C. fulvum*							
	$A_1A_2A_3a_4$		$a_1a_1A_3a_4$	$a_1a_2a_3A_4$	$A_1A_2A_3A_4$	$A_1a_2A_3A_4$	buffer	
	Fig. 8	Fig. 9B	Fig. 8	Fig. 9B	Fig. 8	Fig. 8	Fig. 8	Fig. 9B
Host relation	S	S	S	R	R	R	–	–
Slope a (min^{-1})	0.052	0.070	0.051	0.157	0.119	0.112	0.048	0.071
Standard deviation s (min^{-1})	0.004	0.004	0.003	0.004	0.003	0.006	0.005	0.003
Intercept b	25	26	29	22	27	27	27	24
Correlation coefficient r	0.98	0.997	0.99	0.98	0.996	0.99	0.98	0.99

Table VI Statistical calculations of the results given in Fig. 10A and 10B.
Tomato variety V 121 (Cf 3). Young and old leaves.

	Physiological races of *C. fulvum*							
	$A_1a_2a_2a_4$		$A_1A_2A_3A_4$		$A_1A_2A_3a_4$		buffer	
	young	old	young	old	young	old	young	old
Host relation	S	S	R	R	R	R	–	–
Slope a (min^{-1})	0.126	0.121	0.285	0.193	0.286	0.211	0.110	0.107
Standard deviation s (min^{-1})	0.020	0.006	0.020	0.018	0.029	0.019	0.013	0.011
Intercept b	29	2	27	1	21	2	26	0
Correlation coefficient r	0.95	0.99	0.98	0.98	0.98	0.98	0.97	0.97

Table VII Observed correlation between resistance in vivo and leakage by selective toxins.

Tomato variety		Physiological races of *Cladosporium fulvum*								
		Indices as given by Kooistra (1964) and Hubbeling (1968)								
		0	1	2	3	4	1.2	1.2.3	2.3.4	1.2.4
		Presumed genotype for toxin production								
		$A_1A_2A_3A_4$	$a_1A_2A_3A_4$	$A_1a_2A_3A_4$	$A_1A_2a_3A_4$	$A_1A_2A_3a_4$	$a_1a_2A_3A_4$	$a_1a_2a_3A_4$	$A_1a_2a_3a_4$	$a_1a_2A_3a_4$
Moneymaker Leaf Mould Resister	cf	SN	SN	S	S	SN*	SN	S	S	SN*
No. 1	Cf 1	R	SN	RL	R	R	S	S	R	S
Vetomold	Cf 2	RL*	RL	SN	R	RL	S	SN	S	S
V 121	Cf 3	RL	R	R	S	RL	R	S	SN	R
Purdue 135	Cf 4	RL	R	RL	R	SN	R	RL	S	SN
V 473	Cf 1 Cf 2	RL	RL	R	R	R	SN	S	R	SN
Vagabond	Cf 2 Cf 4	RL*	R	R	R	RL	R	RL	S	SN*

R = Resistant, no leakage experiments;
S = Susceptible, no leakage experiments;
L = Leakage;
N = No leakage.
* Not described in this dissertation, but results were similar to those described for the other combinations tested.

only a minor part of the total extracellular material is involved in the leakage process. Since these host specific compounds are supposed to be excreted by the fungus in vivo in immediate contact with host membranes, it is not surprising that a relatively high concentration of the material has to be infiltrated into discs to evoke an effect in our model experiments.
In Chapter III the experimental data from the inoculation experiments have been described. Hypersensitivity is first witnessed by a strong chlorosis of the infected area. The resistance reaction governed by the Cf 2 and Cf 4 genes is more locally confined than that by the genes Cf 1 and Cf 3. Cf 2 and Cf 4 are both epistatic over Cf 1 and Cf 3 (*cf.* Quadt, 1953; Persiel, 1967). Our results suggest that the genes Cf 2 and Cf 4 evoke an earlier response to the toxins from the incompatible races of *C. fulvum* than the genes Cf 1 and Cf 3. Labelling with ^{32}P has proved a very satisfactory method for studying leakage. However, experiments with ^{36}Cl, like ^{32}P a strong β-emittor, were unsuccessful because no difference could be observed between the incompatible and the compatible combination of host variety and physiological race of the fungus. Even in the buffer control the radioactivity of the solution reached a constant level already very shortly after the infiltration was completed. Chlorine is mostly present in the cell as a single ion whereas phosphorus is involved in many biochemical processes and is bound to larger molecules. Apparently, host cells quickly loose chloride ions upon slight disturbances during infiltration. Hence no differences can be observed with the ^{36}Cl technique.
At present, it is difficult to indicate how the membrane damage occurs. More careful studies with the use of other techniques, *e.g.* the electron-microscopic autoradiography are needed to explain this phenomenon.

E Summary

Model experiments were carried out with the tomato varieties Moneymaker (no resistance gene), Leaf Mould Resister Nr. 1 (resistance gene Cf 1), Vetomold (resistance gene Cf 2), V 121 (resistance gene Cf 3), Purdue 135 (resistance gene Cf 4), V 473 (resistance genes Cf 1 Cf 2) and Vagabond (resistance genes Cf 2 Cf 4) and various physiological races of *C. fulvum* tabulated in Table VII.
Leakage of ^{32}P-labelled compounds from tomato leaf discs was obtained on infiltration with high molecular weight excretion products from incompatible races of *C. fulvum* but not with those from compatible races. Statistical calculations revealed a significant difference between leakage experiments of compatible and incompatible combinations of plant and fungus. It is suggested that the toxic products from the fungus cause specific membrane damage in the incompatible host. These products were obtained by Sephadex G-25 gel filtration of culture filtrates. These fungal products loose activity beyond a 1:2 dilution. Some properties of the active fraction from the gel filtration over Sephadex G-25 are given.
The observations support our hypothesis that the gene-for-gene relation existing between tomato and *C. fulvum* is based on interaction of specific fungal excretion products with specific receptors in the host, presumably in the plasma membrane. The presence of these fungal compounds is supposed to be controlled by four avirulence genes (A_1, A_2, A_3 and A_4), while the four genes for resistance (Cf 1, Cf 2,

Cf 3 and Cf 4) are suggested to control the presence of specific receptors in the cell membranes.

Chapter V

Cessation of fungal growth following the hypersensitive reaction. The possible significance of tomatine

A Introduction

As put forward in the preceding chapter leakage of host cells is a very likely explanation of the hypersensitive reaction in the incompatible combination; however, the occurrence of this reaction does not explain why growth of the pathogen is only very limited in such plants. We know that in the case of apple scab a fungitoxic compound is released after decompartmentalization of the host cells in the incompatible combination of apple and *Venturia inaequalis*. This compound kills the invading fungus (Raa, 1968 a, b) as was also explained in Chapter I. In tomato plants the glycoside tomatine constitutes approx. 0.5–1 % of the dry weight of the leaves. Since this compound is highly toxic to some fungi (Fontaine et al., 1948; Irving, 1947), it seemed possible that it might leak from host cells into the fungus and thus cause cessation of fungal growth in incompatible tomato plant. In the literature no data were found to be available on the fungitoxicity of tomatine to *C. fulvum*.

Kern (1952) made a very thorough study of the possible significance of tomatine in tomato diseases. He concluded that tomatine does not play a rôle in the resistance reaction to *Fusarium oxysporum* f. *lycopersici* of different tomato varieties but suggested the possible rôle of this compound in the resistance of tomato to *C. fulvum*.

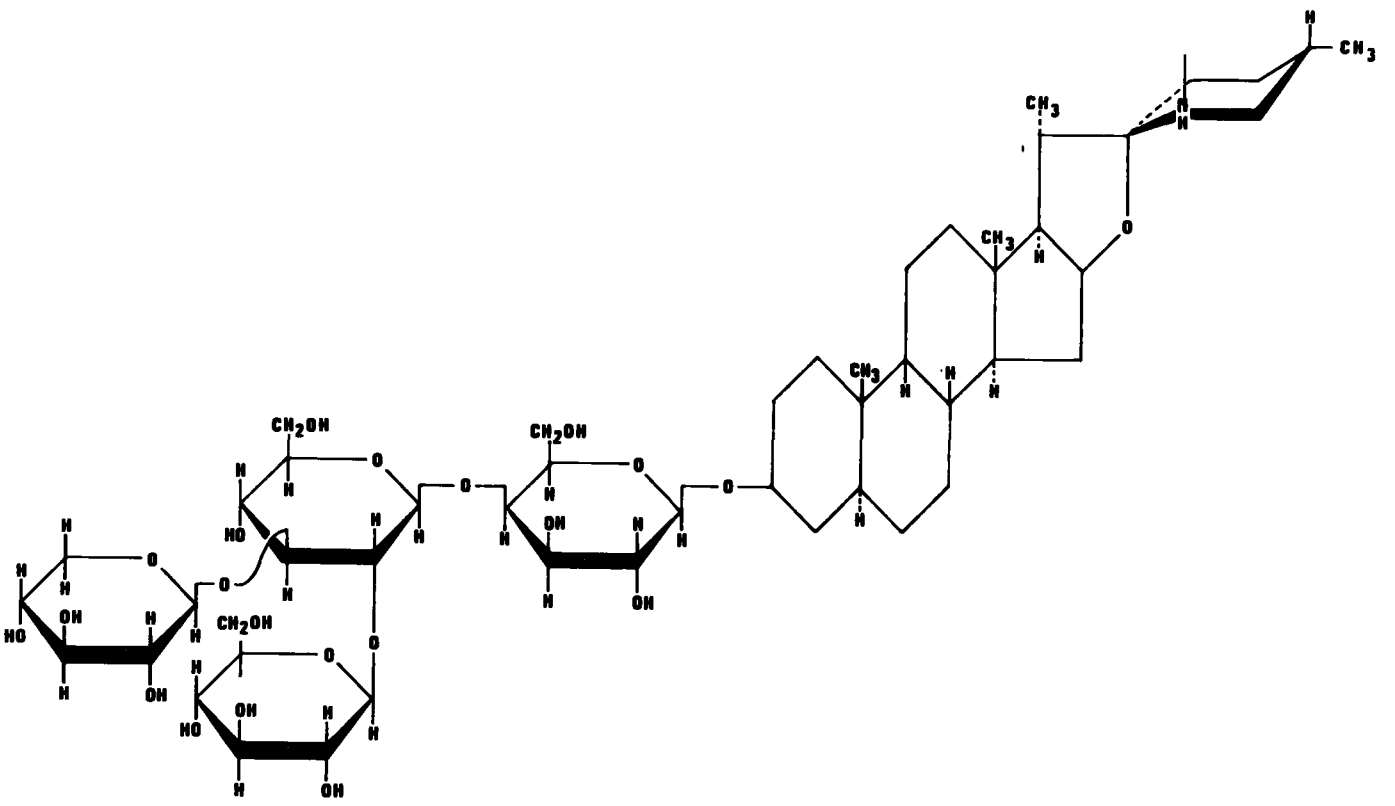
Kühn et al. (1950) supposed that tomatine and other related compounds like solanine, which all have a very bitter taste, might act as a defence mechanism against Colorado beetles since plants with a high content of such compounds were not eaten by them. The steroid-alkaloid glycosides are widely spread among the Solanaceae. Potato tissue contains the glycosides α-solanine and α-chaconine which proved fungistatic to 9 fungi tested (cf. Allen and Kuc, 1968), however, among these were no fungal pathogens of potato.

Arneson and Durbin (1967) studied the relation between the resistance of tomato to three species of Septoria and the sensitivity of these species to tomatine. An aqueous extract of leaves of the tomato variety Bonny Best inhibits spore germination and mycelial growth of *Septoria linicola* and *S. lactucae*, two non-pathogens of tomato, but not of *S. lycopersici*, a tomato pathogen. The toxic substance appeared to be either tomatine or tomatidine.

Water extracts of leaves which had been infected with *S. lycopersici* were not toxic to *S. linicola* and *S. lactucae*. From this fact and the observation that after incubation of tomatine with culture filtrates of the tomato pathogen *S. lycopersici* the liquid was no longer toxic to *S. linicola* and *S. lactucale*, they concluded that *S. lycopersici* is able to detoxify tomatine. Enzyme denaturation destroyed the detoxification mechanism. It was proved that tomatine is hydrolyzed by a constitutive, extra-cellular enzyme which removes one glucose molecule from the tetrasaccharide. The authors assumed that the ability to overcome the toxicity of α-tomatine is necessary for the success of these fungi to act as tomato pathogens. It seems very doubtful whether such an exclusive rôle can be ascribed to tomatine because pathogenicity may be primarily determined by quite different factors.

Neither the work of Irving (1947) and Fontaine et al. (1948) nor the work of Kern (1952) takes into account the possible rôle of conversion products of tomatine as fungicides. In the resistance of some plants

Fig. 1 Structure and nomenclature of α-tomatine



3 β-[[O-β-D-glucopyranosyl-(1 → 2)-O-[β-D-xylopyranosyl-(1 → 3)]-O-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranosyl]oxy]--5α-tomatidane.

to certain diseases it was shown that substances, which are not toxic in the glycoside form, yielded a fungitoxic substance upon enzymatic hydrolysis. Some examples are trichocarpin (Loeschke and Butin, 1961), arbutin (Hildebrand and Schroth, 1964) and tulipaline (Beyersbergen, 1969; Tschesche et al., 1969). It lookes like a masking of the fungicide by the sugars. On the contrary, the aglycon of the glycoside avenacine, a glycoside of a triterpenoidal saponine present in oats, is less fungitoxic to *Ophiobolus graminis* and other fungi tested than the intact glycoside. *O. graminis* var. *avenae*, a pathogen of oats, excretes a very specific glycosidase, avenacinase, which detoxifies the glycoside. *O. graminis* does not produce this enzyme (Turner, 1961). We have seen in Chapter I that in the case of apple scab a very labile fungitoxic compound is formed by oxidation of phloridzin following decompartmentalization in the incompatible host cells (Raa, 1968 a, b).
In view of the fact that tomatine is a potent fungicide to some fungi it seemed worthwhile to investigate its possible rôle in the cessation of growth of *C. fulvum* in incompatible tomato plants. Also decomposition products of tomatine should be considered. The possibility that conversion products of tomatine also might be involved in the cessation of growth of *C. fulvum* had to be considered as well.

B Materials and methods

1 *Plant and fungal material*
The tomato varieties and the different races of *C. fulvum* used in this chapter have already been described in Chapters III and IV. In some experiments the wild Lycopersicon species *L. hirsutum* and *L. pimpinellifolium* were included. The seeds of these races were also supplied by the Institute for Horticultural Plantbreeding (I.V.T.).

2 *Chemicals and enzyme preparations*
Tomatine was purchased from Fluka A. G. and tomatidine was obtained by acid hydrolysis of tomatine. From 2.5 g of pure tomatine 1 g of pure tomatidine was obtained. The purity was checked by R_F-value on thin layer plates and melting point. The preparation was carried out as follows: Hydrolysis in 1 N HCl (4 h, 100°C), after cooling and precipitation with NaOH the precipitate was redissolved in methanol. This yielded after evaporation a greyish powder which was dried over P_2O_5 (Sato et al., 1951). The enzymes lipase (0.1 E.U./mg), papain technical grade and trypsin (0.54 Anson u./g) were from BDH, Poole, England. They were purified over Sephadex G-25 fine grade to remove all low molecular weight material.

3 *Extraction of crude tomatine from leaves*
Tomato leaves were dried in an oven at 60°C, then crushed and extracted with 2% acetic acid (v/v). After 48 hours the material was filtered through two layers of cheesecloth. The clear solution was made alkaline with NH_4OH and the resulting precipitate was centrifuged, washed with water and finally taken up in methanol. After evaporation the solution yielded a grey powder. Further purification was not performed.

4 *Preparation of a cell-free extract from tomato tissue*
Cell-free sap of tomato tissue was obtained by a specially designed press. Leaves were pressed between two PVC discs with a pressure of 10 metric

tons on 56.5 cm^2.
The sap was collected and immediately centrifuged in the cold (5 min., 10.000 x g at 4°C), thereafter the supernatant was sterilized by membrane filtration (filter pore Ø 0.8 μ).

5 *Preparation of soluble protein fraction of tomato leaf tissue*
A cell-free preparation of tomato leaves was obtained after crushing leaf tissue in a Tris-HCl buffer pH 6 in a Biox-Press (LKB, Sweden) at –30°C. The resulting homogenate was centrifuged at 14.000 x g for 30 min. in the cold. The clear supernatant was applied to a Sephadex G-25 five grade column and the V_0-fraction was collected and freeze-dried. A white powder resulted.

6 *Detection of fungitoxic products*
Thin-layer chromatography was performed with Merck Silica thin-layer plates on glass or aluminium sheets with butanol: acetic acid: water 4:1:5 v/v, upper layer. Detection of tomatine and other toxic products occurred with the fungicide – bioassay described by Dekhuijzen (1961) or with the specific reagents on steroids: 20% phosphormolybdic acid in ethanol or $SbCl_3$ in glacial acetic acid (1 to 1, w/w). $SbCl_3$ gives pink to purple spots after drying and phosphormolybdic acid gives green to dark-blue spots after heating up to 110°C. Most of the colours disappear after some days.

7 *Fungicidal test*
To asses fungitoxicity the common agar plate test, the shake culture test, the spore germination test as well as the roll culture method, as described by Pluijgers and Kaars Sijpesteijn (1966), were used. The concentrations applied always ranged from 1, 2, 5, 10 . . . 2000 ppm. Tomatine and tomatidine were sterilized by autoclaving in the medium. Chromatography on thin layer plates proved the stability of the compounds to autoclaving.

C Results

1 *Fungitoxicity of tomatine and tomatidine*
a *Roll culture tests.* Fungitoxicity towards three physiological races of *C. fulvum* was estimated with the routine roll culture method. Table I shows that no activity exists within this concentration range. Sensitivity of the following fungi to tomatine and tomatidine was determined as well: *Botrytis allii, Penicillium italicum, Aspergillus niger, Cladosporium cucumerinum, Glomerella cingulata* and *Rhizopus nigricans* (Table II).
An agar plate test carried out with the same fungi was in good agreement with the results of the roll culture method.

b *Spore germination tests.* For *C. fulvum* race $A_1A_2A_3A_4$ also spore germination tests were carried out (Table III.) After 48 hours all spores had germinated; there were no differences between the control and the highest concentrations of the compounds. In the tomatine experiment this compound was still present at the end of the test. Results with germinated conidia did not differ from those with ungerminated spores. Viable germinated conidia had developed extensive hyphae during 24 h in the presence of the compound.

c *Shake culture tests.* In addition to the above mentioned experiments shake cultures of several

Table I Minimum inhibitory concentration (ppm) of tomatine and tomatidine for conidia of three races of *C. fulvum*. Medium: potato dextrose agar (pH 6). Incubation time 11 days at 24° C.

	$A_1A_2A_3A_4$	$A_1A_2A_3a_4$	$A_1a_2a_3a_4$
Tomatine	> 1000	> 1000	> 1000
Tomatidine	> 1000	> 1000	> 1000

Table II Minimum inhibitory concentration (ppm) of tomatine and tomatidine for 6 different fungi in glucose agar (pH 6) incubated 3 days at 24° C.

	B. alii	*P. italicum*	*A. niger*	*R. nigricans*	*C. cucumerinum*	*G. cingulata*
Tomatine	100	20	> 500	> 100	10	10
Tomatidine	> 500	> 500	> 500	> 100	> 500	> 100

Table III Percentage germination of conidia of *C. fulvum* race $A_1A_2A_3A_4$ in tomatine and tomatidine after 24 and 48 h at 24° C.

		% germinated spores			
		tomatine		tomatidine	
		24 h	48 h	24 h	48 h
Concentration in ppm	0	80	100	80	100
	20	82	100	57	100
	50	54	100	63	100
	100	51	100	48	100

physiological races of *C. fulvum* were incubated with 1000 ppm of tomatine or tomatidine. Growth was determined by the mycelium dry weights after 14 days (Table IV).

The data from the table show little or no growth inhibition at 1000 ppm tomatine or tomatidine.

The fungicidal tests listed above show clearly neither tomatine nor tomatidine inhibits growth of the different races of *C. fulvum* even at 1000 ppm. To study the effect of pH on the fungitoxicity of tomatine, plate and shake cultures were prepared with pH 3.2 – 4.6 – 5.2 – 5.5 – 5.9 – 6.5 – 7.8 and a tomatine content of 0 and 1000 ppm in the basal medium. *C. fulvum* $A_1A_2A_3A_4$ and $A_1a_2a_3a_4$ were tested on these media. Again, no growth inhibition was found at any of the different pH's.

Quantitative extractions of tomatine from the variety Beauty, carrying the Cf 2 and Cf 4 genes for resistance and the variety Moneymaker (no genes for resistance) was performed.

From 160 g dried leaves of the susceptible plant approx. 760 mg tomatine was obtained i.e. 0.48% of the dry weight. The dried leaves of the resistant plant contained 0.52%. These findings are in good agreement with those of Kern (1952). We determined also the tomatine content of the following tomato varieties: Leaf Mould Resister No. 1, Vagabond, Vetomold and the wild species *L. pimpinellifolium* and *L. hirsutum*. These experiments were in line with our first findings.

According to Kern (1952) and our own observations the tomatine content of tomato leaves varies from 0.5 – 1%. Solubility of tomatine is very poor. At pH 7 a saturated solution contains about 230 ppm. So testing higher concentrations > 1000 is not well possible.

Table IV The effect of 100 ppm tomatine or tomatidine on the growth of several races of *C. fulvum*. Medium: Basal shake culture medium, pH approx. 6. Incubation time 11 days at 24° C.

Races of *C. fulvum*	Mycelium dry wt. in g.		
	Control	1000 ppm tomatine	1000 ppm tomatidine
$A_1A_2A_3A_4$	1.04	1.14	0.77
$a_1A_2A_3A_4$	0.17	0.15	0.14
$A_1a_2A_3A_4$	0.19	0.19	0.33
$a_1a_2A_3A_4$	0.20	0.21	0.37
$a_1a_2a_3A_4$	0.71	0.63	0.44
$A_1A_2A_3a_4$	0.28	0.27	0.40
$A_1a_2a_3a_4$	1.08	1.05	1.28

2 *Growth on cell-free sap of tomato*

The ability of *C. fulvum* races $A_1A_2A_3A_4$ and $A_1A_2A_3a_4$ to grow on cell-free leaf extracts of the wild tomato *Lycopersicum hirsutum* and of the susceptible tomato *L. esculentum var.* Moneymaker was established. Undiluted sap, sterilized by membrane filtration (5 ml in a 15-ml flask) of tomato leaf tissue appeared to be a satisfactory shake culture medium for these two races of *C. fulvum*. No growth inhibition was observed on thin medium as compared with growth on the shake culture media used in Chapter III. A similar experiment was carried out with the following tomato varieties: Moneymaker, Vetomold and Vagabond, and the physiological races $A_1A_2A_3A_4$ and $A_1a_2a_3a_4$ of *C. fulvum;* in all cases good growth was witnessed in spite of the fact that Vetomold is resistant to one race and Vagabond to both races.
The time interval in this experiment between the sterile filtration of the sap and the addition of fungal spores was 1.5 h. Since a very labile fungitoxic product formed on homogenization may have disappeared already before spore germination occurred the experiment described above was repeated with germinated spores: At different times (0, 2, 5, 10 and 30 min.) after the filtration procedure was finished, germinated conidia were suspended into approx. 5 ml sterile (filtered) sap of the same tomato varieties as used in the previous experiment. The whole procedure was carried out at room temperature. There was no visible difference in growth in the various flasks.
Neither did we obtain any indication for a fungitoxic product being formed after homogenization: the formation of a transient fungicide formed from tomatine in a homogenized tissue, which situation is even more comparable with that after

decompartmentalization takes place in an incompatible combination of plant and fungus, was also investigated. It was even found that cell-free juice as well as homogenates of tomato leaf tissue provide an excellent growth medium for all races of *C. fulvum* tested.
Decomposition of tomatine in leaf homogenates was also investigated. Tomato leaves were homogenized in a Waring Blendor with water (1:1, wt/vol.) and 0.1 ml samples were taken at 0, 1, 2 and 18 hours after homogenization. The samples were chromatographed in duplicate and on thin layer plates treated with phosphormolybdic acid or bioassayed with *Cladosporium cucumerinum*.
Due to the slow mycelial growth of *C. fulvum* this fungus could not be used for assaying fungitoxic products on the chromatograms. The other chromatograms showed that tomatine is decomposed into tomatidine and products with a higher R_F-value than tomatidine; with the exception of tomatine no compounds toxic to *C. cucumerinum* were observed. The rate of decomposition was equal for all tomato varieties tested. Thus, no correlation between the rate of break-down and resistance was found.

3 *Decomposition of tomatine by fungi*
All physiological races of *C. fulvum* available were checked for their ability to decompose tomatine by determination of the presence of tomatine and tomatidine in the fungus and in the culture filtrate by thin-layer chromatography.
By incubation overnight heavy mycelial suspensions of all races of *C. fulvum* completely transformed 1000 ppm tomatine, tomatidine being the main product formed. Race $A_1A_2A_3A_4$ and race $A_1a_2a_3a_4$ which grown very well, both decomposed tomatine completely in a two week growth period. 2000 ppm in 125 ml shake culture medium were seeded with a spore suspension.
The supposition made by Arneson and Durbin (1967) that pathogens of tomato can break down tomatine whereas non-pathogens can not, was checked by the same technique.
Spores of the non-pathogens. *B. allii, P. italicum, A. niger, R. nigricans* and *C. cucumerinum* were incubated for 10 days in shake cultures containing 2000 ppm tomatine. All fungi tested were able to decomposed tomatine as shown by Table V. Some differences in the speed of the decomposition was observed. *C. cucumerinum*, which after three days shows no visible growth on 10 ppm tomatine present in the medium, showed the slowest rate of tomatine break-down.
As we have seen above, the physiological races of *C. fulvum* are able to degrade tomatine. The same was found with regard to *F. oxysporum* f. *lycopersici* (Langcake et al., 1972) and for *S. lycopersici* (Arneson and Durbin, 1967; Durbin and Uchytil, 1969).
These experiments show quite clearly that for the fungi examened no correlation between pathogenicity to tomato and the ability to degrade tomatine does exists. Table V summarizes the results and shows the relation with the fungitoxicity of tomatine as tested in vitro.

4 *Influence of tomatine on the permeability of tomato cells*
The experiments described in Chapter IV furnish strong evidence for the view that the hypersensitive reaction is preceded by the loss of

Table V Relation between pathogenicity on tomato, the fungitoxicity of tomatine and the ability to decompose tomatine of several fungi.

	Tomato pathogen	M.I.C. of tomatine	Ability to decompose tomatine
C. fulvum	+	> 1000	+
S. lycopersici	+	> 1000	+
F. oxysporium f. lycopersici	+	> 1000	+
S. linicola	−	> 100	
S. lactucae	−	> 100	
B. allii	−	100	+
P. italicum	−	20	+
A. niger	−	> 500	+
R. nigricans	−	> 100	+
C. cucumerinum	−	10	±

semipermeability and leakage of host cell tissues. The question arises whether the tomatine present in the cells contributes to this leakage after its release has been triggered by toxic fungal excretion products. It has been reported that tomatine is released by decompartmentalization (see Chapter IV, section C2). Moreover, steroid-alkaloids are known to disturb semi-permeability of membranes (Ferenczy and Kevei, 1966; Child et al., 1969).
The following experiment was carried out to establish whether tomatine or tomatidine can influence the permeability of membranes of tomato tissue. Two mm sections of stem tissue (2 g) were incubated with approx. 300 ppm tomatine or tomatidine in water. Every 15 min. leakage of electrolytes through the membranes of tomato cells was determined by measuring the conductivity of the solution with a 3-ml conductivity cell connected with the Phyloscoop. The increase of conductivity for the resistant and the susceptible tissue were identical and did not differ from that of the water control. Consequently, externally added tomatine and tomatidine does not influence permeability.

5 *Other suggestions for the cessation of growth of C. fulvum in incompatible combinations*
In our search for the mechanism which kills the penetrating hyphae in incompatible combinations of tomato and *C. fulvum* the possibility that enzymes released by the plant would exert a destructive activity on the fungus was investigated. High amounts of potent proteinases and lipases might be able to hydrolyze the extracellular enzymes of the fungus, or the protein or lipid structures of the plasma membranes of the fungus.
Viable germinated spores were incubated with 0, 10,

20, 50, 100, 200 and 500 ppm of a purified preparation of either papain, an endopeptidase from papaya fruits, trypsine or lipase. No influence on germination of the spores or growth of the hyphae of the races $A_1A_2A_3A_4$, $a_1a_2a_3A_4$ and $a_1a_2A_3a_4$ of *C. fulvum* was observed. The same experiment was undertaken with a soluble protein preparation containing per ml 10 mg of the protein preparation in 0.01 M phosphate buffer of pH 6 from three different varieties of tomato *viz.* Vagabond (Cf 2, Cf 4), Vetomold (Cf 2) and Moneymaker (no genes for resistance). No effect was observed on growth of the hyphae emerging from the spores of the three races of *C. fulvum*.
From these results we concluded that it is unlikely that plant enzymes can be held responsible for the cessation of growth of *C. fulvum* in incompatible combinations.
The only possibility left is a deduction made from a macroscopical observation in our inoculation experiments. Following the destruction of the cell structure, caused by the incompatibility reaction of plant and fungus, the invaded tissue quickly starts to loose its water, resulting in a dry spot. We know already that *C. fulvum* requires a relatively high humidity for growth and since no other effect has been found so far, drying out of the invading hyphae is at present the most plausible explanation for the cessation of growth of *C. fulvum* in incompatible combinations.

D Discussion

The experiments described have shown that at a 1000 ppm level neither tomatine nor tomatidine is directly fungitoxic or fungistatic to any of the physiological races of *C. fulvum*. Moreover the content of tomatine in leaves of the different varieties was found to be almost equal: 5000 – 10.000 ppm tomatine per dry weight which is approx. 500 – 1000 ppm per wet weight. To test fungitoxicity of higher concentrations was useless due to the poor solubility of tomatine.
Other possible suggestions for the cessation of fungal growth were submitted to investigation. But the experiments described did not give any positive information.
Permeability changes are not evoked by contact with tomatine or tomatidine. A mechanism to accelerate the hypersensitive reaction cannot be ascribed to tomatine therefore. No compound fungitoxic to *C. fulvum* was found in sap or in homogenized tomato tissue. Neither were unstable fungitoxic intermediates detected.
The most plausible mechanism at present is the drying out of the invaded area. Further investigations are required to elucidate the cause of fungal death.
The views put forward by Arneson and Durbin (1967) that tomato plants are resistant to *S. lactucae* and *S. linicola* because these cannot detoxify tomatine, and that tomato plants are susceptible for *S. lycopersici* because this fungus can overcome the toxicity of tomatine, are untenable. Should the inability of *S. linicola* and *S. lactucae* to break down tomatine be an explanation for the resistance of tomato to these two fungi, then other fungi which can break down tomatine might also be expected to be a tomato pathogen (e.g. *Aspergillus niger*).
Futhermore, it is questionable in how far one can compare these three fungi imperfecti. They have only tentatively been given the same genus name: Septoria. Real proof for a relationship has not been given. The

same arguement applies to the work of Allen and Kuc (1968). They reason that potato is resistant to *Helminthosporium carbonum* because this fungus is sensitive to the steroidalkaloid α-chaconine present in potato. No potato plant is known, however, which is susceptible to *H. carbonum*.
The most fruitful way to investigate mechanisms of resistance may be the study of the differences of closely related resistant and susceptible varieties of the same plant species, *e.g.* apple-*V. inaequalis* and tomato-*C. fulvum*.
The proposed relationship between tomatine content of tomato and resistance against *C. fulvum* as pointed out by Kern (1952) could not be established; neither is the supposition of Arneson and Durbin (1967) convincing that non-pathogens of tomatoes are not parasitic because they are unable to hydrolize tomatine.
We found that pathogens as well as non-pathogens can hydrolyze tomatine. No correlation could be observed.

E Summary

The possible significance of tomatine or its conversion products for the natural resistance of tomato to *C. fulvum* was investigated. Tomatine did not prove to be fungitoxic to *C. fulvum*, neither were fungitoxic conversion products formed. Therefore no rôle could be ascribed to these compounds for the cessation of fungal growth following the hypersensitive reaction of tomato to *C. fulvum*. Other suggestions were investigated, but none of these can be held responsible for the death of the fungus in incompatible combinations. This is in contrast to the findings in the hypersensitive reaction of apple leaves to *V. inaequalis*. Until now the physical mechanism of drying out seems the most plausible explanation for the cessation of fungal growth. The proposed correlation between the ability of a fungus to hydrolyze tomatine and the pathogenicity on tomato as suggested in the literature could not be confirmed.

Chapter VI

Polyacrylamide disc gel electrophoresis of the Sephadex G-25 V_0-fractions of culture filtrates from C. fulvum races

A Introduction

Disc gel electrophoresis of soluble proteins of fungal species has already been employed successfully as a diagnostic and taxonomic aid by several workers in the phytopathological and mycological field. A taxonomic approach with this technique was carried out with the genes Phytophthora (Hall et al., 1970; Gill and Powell, 1963; Clare, 1963), Septoria (Durbin, 1966) and Ceratocystis (Stipes, 1967). Gel electrophoresis proved to be more convenient than the serological method based on immunological reactions, although this latter method has other advantages.

Recently, electrophoretic separation of proteins in polyacrylamide gels showed differences between two pathotypes of *Heterodera rostochiensis* (Trudgill and Carpenter, 1971). The technique is now frequently used in studying changes in protein and enzyme patterns in host-parasite interactions (Stavely and Hanson, 1967; Wheeler et al., 1971; Van Loon and Van Kammen, 1968). Because of its high resolution the disc electrophoresis technique is useful for the separation of complex protein mixtures into a pattern of characteristic bands.

Gill and Powell (1968) obtained protein patterns by this technique of 17 isolates of 8 physiological races of *Phytophthora fragariae*. These were nearly identical. Races or biotypes of *P. fragariae* could not be differentiated.

Shipton and Fleischmann (1969) found no differences in the protein patterns obtained by disc gel electrophoresis on polyacrylamide of extracts of soluble proteins from uredospores of *Puccinia coronata* f. *avenae*.

On the other hand Macko et al. (1967) claimed that the electrophoretical patterns of uredospore protein of two physiological races of *Puccinia graminis* f. *tritici* differed significantly. Thus it appears that little information is available on differences in the number and/or position of protein bands in different physiological races of pathogens.

Although differences between physiological races have been associated with differences in the electrophoretical protein pattern, evidence for this phenomenon is still scarce. It was thought valuable to apply disc gel electrophoresis to the soluble proteins of culture filtrates of *Cladosporium fulvum*.

In Chapter IV we have described the preparation of high molecular weight material from different physiological races of *C. fulvum* by Sephadex G-25 gel filtration. The preparation of the different races showed characteristic properties in our leakage experiments. The availability of races of *C. fulvum* isolated on different places in The Netherlands, but possessing the same set of avirulence alleles, enabled us to carry out comparative disc gel electrophoresis on polyacrylamide gels. The results of these experiments and an evaluation of this technique will be described in this chapter.

B Materials and Methods

1 *Fungal material and cultural techniques*

Many details have already been given in Chapters III and IV.

The isolates of *C. fulvum* with the same genotype for avirulence were collected at different sites in The Netherlands, some of them more than 100 km away from each other. The isolates used throughout this study are tabulated in Table I which gives the

genotypes for avirulence, the indices for pathogenicity according to Kooistra (1964) and Hubbeling (1968) and the place of isolation.
The techniques of shake cultures and the production of high molecular weight material from the culture filtrates have been described in Chapter IV.

2 *Chemicals*
Acrylamide – cyanogum 41 (British Drug Houses) and N_1N' – methylene bis acrylamide (BIS, British Drug Houses) were purified by recrystallization from chloroform at 50°C and acetone at 40°C, respectively, according to Loening (1967). All other chemicals used were reagent grade.

3 *Electrophoresis of the soluble extracellular proteins*
Disc gel electrophoresis of the soluble extracellular proteins from the V_0-fractions was performed according to Ornstein (1964) and Davis (1964) with slight modifications.
The polyacrylamide gels were prepared in running-tubes 5 mm I.D. x 75 mm as described by Davis (1964), but we used the persulphate catalyst system for both spacer and separation gels. The concentration of the poly-acrylamide was $7^1/_2$%. Electrophoresis was performed at pH 9.5 and approx. 4°C in a commercial apparatus (Shandon Scientific Company, London) during 2.5 min at 1 mA per gel followed by 45 min at 5 mA per gel.
Samples from the different freeze-dried high molecular preparations were dissolved in the buffer solution of the spacer gel and centrifuged at 10.000 g for 10 min at 4°C. Aliquots from 5 to 100 µl containing 0.1 to 2.3 mg protein from these solutions were applied on top of the spacer gels. It was not possible to apply for the several races the same

Table I *List of the physiological races and isolates of C. fulvum* used in the present investigation.

Derived genotype for avirulence	Indices[1]	Locality of isolation	Province
$A_1A_2A_3A_4$	0	Arcen*	Limburg
$A_1A_2A_3A_4$	0	Dordrecht	Z-Holland
$a_1A_2A_3A_4$	1	Naaldwijk*	Z-Holland
$A_1a_2A_3A_4$	2	Sappemeer*	Groningen
$A_1A_2a_3A_4$	3	Utrecht*	Utrecht
$A_1A_2A_3a_4$	4	Den Hoorn	Z-Holland
$A_1A_2A_3a_4$	4	Naaldwijk	Z-Holland
$A_1A_2A_3a_4$	4	Naaldwijk*	Z-Holland
$a_1a_2A_3A_4$	1.2.	Enkhuizen*	N-Holland
$a_1a_2a_3A_4$	1.2.3.	unknown	
$a_1a_2A_3a_4$	1.2.4.	Drunen	N-Brabant
$a_1a_2A_3a_4$	1.2.4.	Wilnis*	Utrecht
$a_1a_2A_3a_4$	1.2.4.	Wellerlooi	Limburg
$A_1a_2a_3a_4$	2.3.4.	Naaldwijk	Z-Holland
$A_1a_2a_3a_4$	2.3.4.	Bergschenhoek*	Z-Holland

[1] Indices as given by Kooistra (1964) and Hubbeling (1968).
* These races were used in our leakage experiments described in Chapter IV.

concentration of protein of the different races on the gels due to variation in the viscosity of the solutions. No front marker, like bromophenol blue, was needed because a magenta coloured pigment running with the front was functioning as such. The gels were removed from the running tubes by irrigation with a needle (Mauer, 1968). After fixation in 12.5% trichloroacetic acid the protein bands were stained with Coomassie Brilliant Blue R 250, a characteristic stain for proteins, in 12.5% trichloroacetic acid as described by Chrambach et al. (1967).
To lower the background effect of this staining procedure the gels were de-stained overnight in 10% trichloroacetic acid before scanning. Densitometer tracings of the gels were obtained by using a Photovolt model 520 – a densitometer with reduced slit width, equiped with Varicord 43 linear/log recorder. A red filter with maximum transmittance at 610 nm was used for these scans. In later experiments we applied a Beckman DU spectrophotometer equiped with a Gilford Gel Scanner model 2411 at 583 nm. Scans were recorded on a Servogor compensation recorder RE 514.

C Results

1 *High molecular weight fractions of culture filtrates of C. fulvum*
The isolates of *C. fulvum* examined are given in Table I. Inoculation experiments confirmed that within the different isolates we were dealing with 9 different physiological races (*cf.* Chapter III and Hubbeling, 1968, 1971).
The freeze-dried V_0-fractions, as described in Chapter IV, were used in the electrophoretical experiments. As was stated in Chapter IV no differences were found in the elution pattern of the culture filtrates. Fig. 1 in Chapter IV illustrates such an elution pattern. Also the pattern of the second purification over Sephadex G-25 was largely identical for the different isolates (Fig. 2, Chapter IV).

2 *Electrophoresis of the V_0-fractions*
The high viscosity of the solution prevented diffusion of the sample into the electrode buffer, so no sucrose was required on the spacer gel.
At the electrophoretical front we always found a magenta coloured band at the pH used (pH 9.5). The band turned orange when the gel was placed in 1 N HCl. The anthraquinone dye cladofulvine, we isolated from *C. fulvum*, when applied to the system under the same electrophoretical conditions had the same electromobility and colour as the coloured band in our V_0-fraction of *C. fulvum* culture filtrates. These findings suggest that the coloured band contains cladofulvine. The electrophoretic protein patterns were determined without staining by measuring the absorption of UV light at 280 nm after fixation for 30 min in 12.5% trichloroacetic acid. No distinct peaks were observed. Staining the gels with Amido blue black according to Davis (1964) revealed a few weak bands. The staining procedure of Chrambach et al. (1967) with Coomassie Brilliant Blue resulted in clear, well-reproducible protein patterns.
No protein bands could be detected after electrophoresis with reversed current. Apparently, all proteins had moved into the separation gel under the electrophoretical conditions normally applied.
Since it is known that the persulphate catalyst can cause artefacts (Loening, 1967), we carried out some control runs without addition of samples. When

immersed in a 2% solution of benzidine in 10% acetic acid, no blue colour developed. Obviously, no persulphate was present in our gels. (*cf.* Bennick, 1968).

3 *Electropherograms of extracellular proteins of the various physiological races of C. fulvum*
Results of all experiments are given diagrammatically in Fig. 12 and partly illustrated by Figs. 1–9. In Figs. 1–9 the striking differences in the electrophoretical patterns of nine different physiological races of *C. fulvum* are shown.
The patterns had a good reproducibility; also at various concentrations and volumina (5 to 100 µl) of the preparations no qualitative variations were detected. However, when samples were too concentrated their high viscosity caused disturbancies in the spacer gel.
Electrophoresis of preparations from culture filtrates of a single isolate of *C. fulvum* grown for different periods showed always the same electrophoretical pattern. Hence we could combine all preparations of one isolate to standardize our techniques.
The electrophoretical pattens obtained from different isolates with the same genotype for avirulence (*cf.* Table I) reveal also differences as appears from comparison of Figs. 10 and 11 and from Fig. 12.

D Discussion

Our data show clearly that there are distinct and reproducible differences in the electrophoretic patterns of the extracellular proteins of 9 different physiological races of *C. fulvum* (Figs. 1–9). In two cases the two patterns from different isolates with

Figs. 1–9 Electropherograms of soluble extracellular proteins of different physiological races of *C. fulvum*. Electrophoresis was performed during 45 min, 5 mA/gel in 7.5% acrylamide gels at pH 9.5, temperature approx. 4° C. The gels were stained with Coomassie Brilliant Blue R 250 and scanned at 583 or 610 nm after one day.

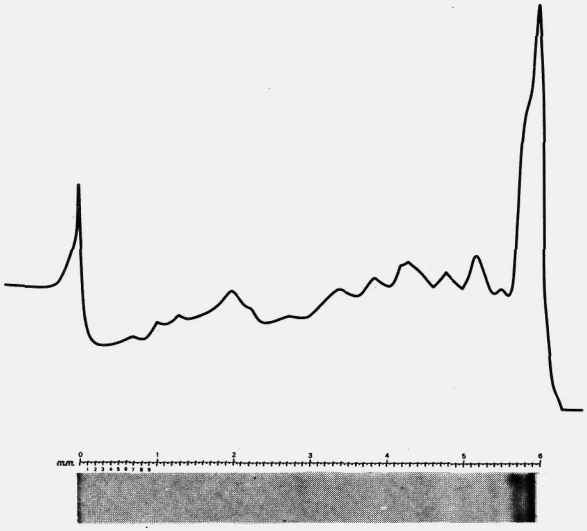


Fig. 1 50 µl aliquot with 0.145 mg protein of physiological race $A_1A_2A_3A_4$, isolate from Arcen.

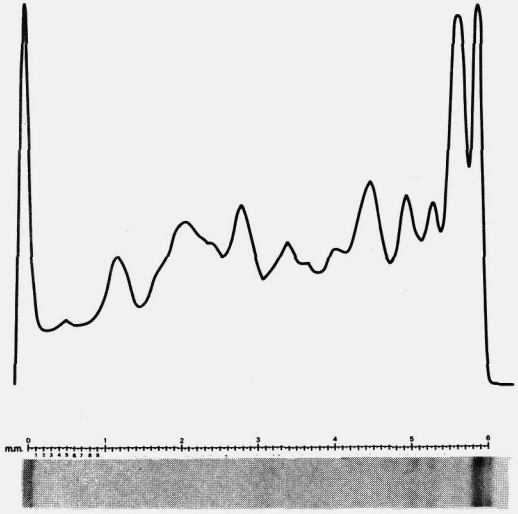


Fig. 2 20 µl aliquot with 0.094 mg protein of physiological race $a_1A_2A_3A_4$, isolate from Naaldwijk.

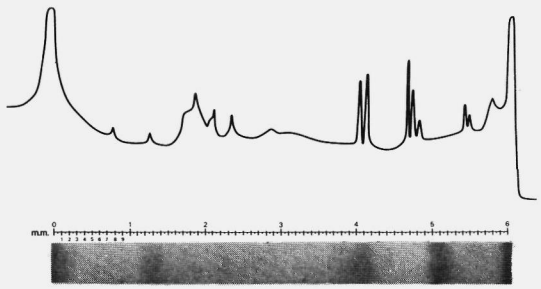


Fig. 3 10 µl aliquot with 0.025 mg protein of physiological race $A_1a_2A_3A_4$, isolate from Sappemeer.

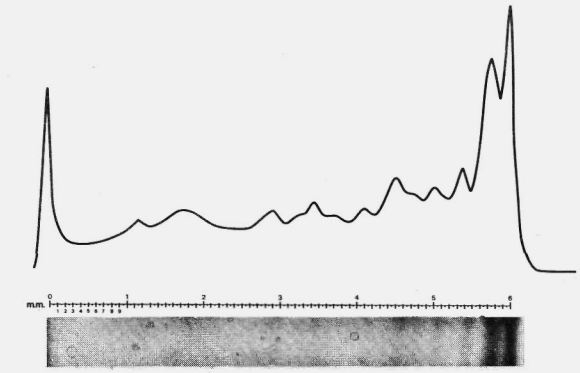


Fig. 5 25 µl aliquot with 0.038 mg protein of physiological race $A_1A_2A_3a_4$, isolate from Naaldwijk.

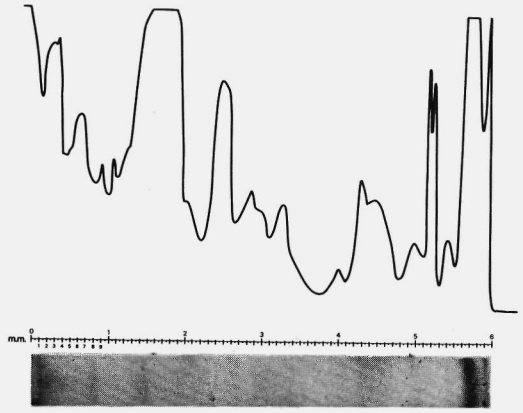


Fig. 4 10 µl aliquot with 0.025 mg protein of physiological race $A_1A_2a_3A_4$, isolate from Utrecht.

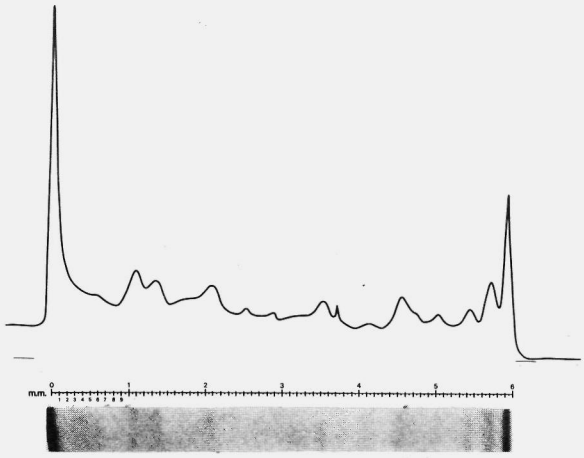


Fig. 6 25 µl aliquot with 0.094 mg protein of physiological race $a_1a_2A_3A_4$, isolate from Enkhuizen.

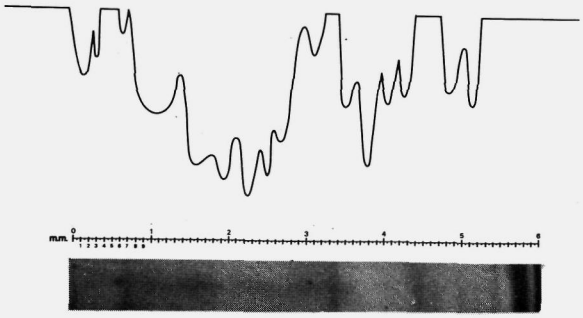


Fig. 7 25 µl aliquot with 0,062 mg protein of physiological race $a_1a_2a_3A_4$, locality of isolation unknown.

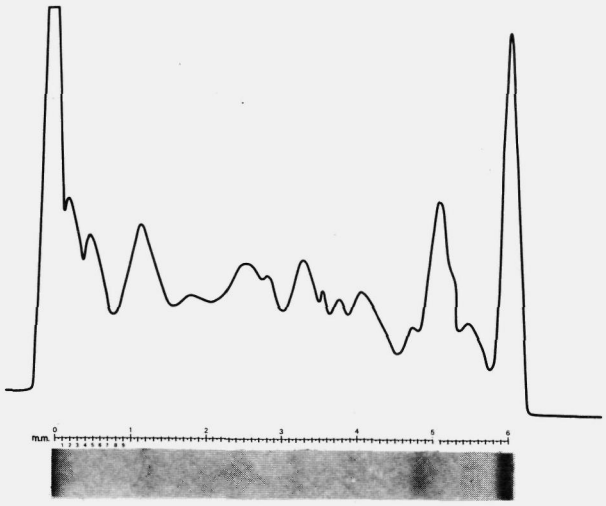


Fig. 8 25 µl aliquot with 0.038 mg protein of physiological race $A_1a_2a_3a_4$, isolate from Bergschenhoek.

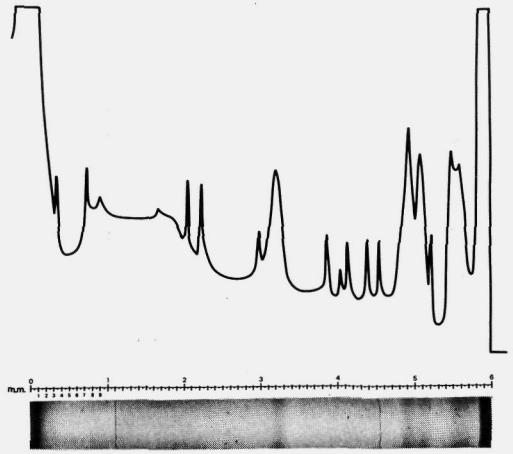


Fig. 9 50 µl aliquot with 0.125 mg protein of physiological race $a_1a_2A_3a_4$, isolate from Drunen.

Fig. 10 Electropherograms of soluble extracellular proteins of 2 different isolates of *C. fulvum* with the same genotype for avirulence.
A: 50 µl aliquot with 0.125 mg protein of physiological race $a_1a_2A_3a_4$, isolate from Drunen;
B: 50 µl aliquot with 0.125 mg protein of the same physiological race isolate from Wilnis.

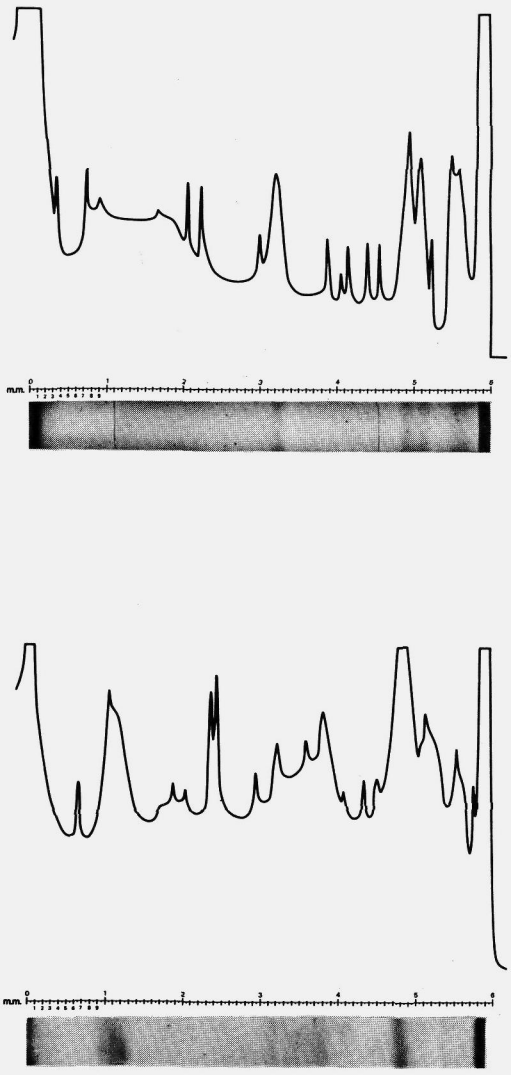


Fig. 11 Electropherograms of soluble extracellular proteins of 2 different isolates of *C. fulvum* with the same genotype for avirulence.
A: 25 µl aliquot with 0.038 mg protein of physiological race $A_1A_2A_3a_4$, isolate from Naaldwijk;
B: 100 µl aliquot with 0.026 mg protein of the same physiological race isolate from Den Hoorn.

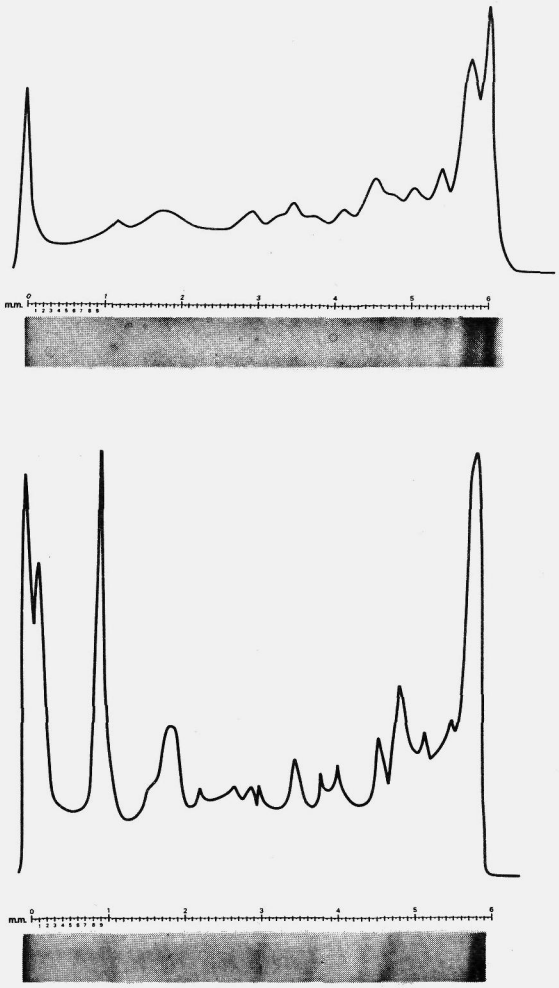
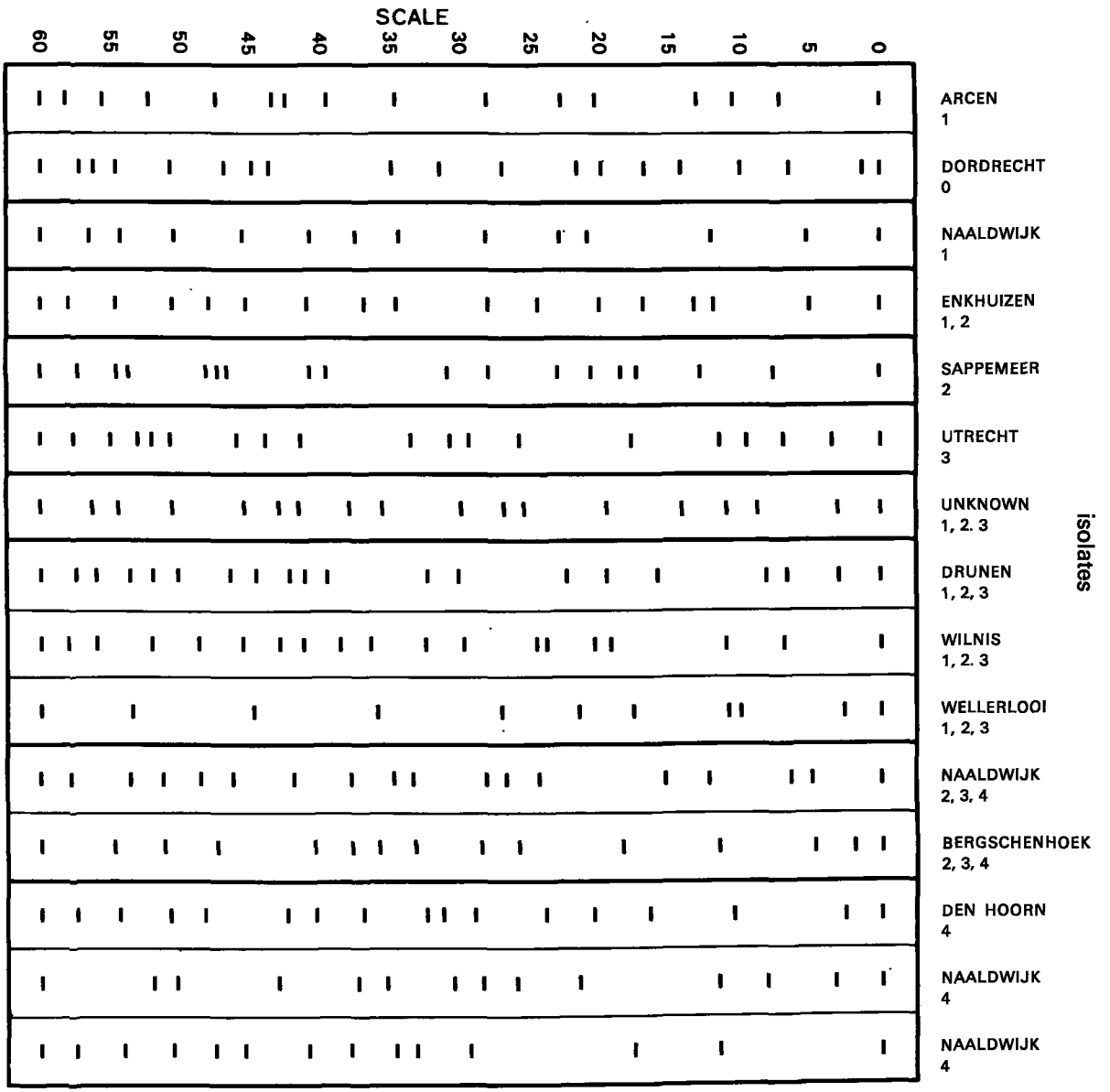


Fig. 12 Diagrammatic presentation of the electrophoretical patterns of soluble extracellular proteins of 15 different isolates of *C. fulvum* (*cf.* Figs. 1–11).



an identical genotype for avirulence genes (Figs. 10–11) were studied. From the figures the conclusion can be drawn that there are differences between the isolates with an identical genotype for avirulence genes. Some bands are corresponding.
With the technique used it was not possible to establish whether any of the protein bands correspond to the compounds elaborated by the four different genes for avirulence, however, in the patterns of different isolates with the same set of avirulence genes one can exclude those bands which are not common in both gels (*cf.* Fig. 12). Quantitative gel electrophoresis will be needed to further elucidate this problem.
The preparations did show biological activity in the leakage tests as described in Chapter IV, and thus fungal products playing a rôle in pathogenicity must have been present. However, no evidence can yet be given that the principle causing leakage in our bioassays is a protein.
Of course many different proteins may be expected to be present in the soluble protein fraction (V_0-fraction) of the culture filtrates of *C. fulvum*, but most of these proteins will not be of any importance in the host-parasite interactions of tomato and *C. fulvum*. The capacity of proteins to bind Coomassie Brilliant Blue varies strongly with character, so the intensity of the stained bands cannot be correlated with the amount of protein present. Obviously not all proteins are necessarily present in quantities large enough to develop a colour with this dye. Moreover, any band, especially the solid ones may be a mixture of different proteins of more or less identical mobilities; therefore it is possible that the products of the avirulence genes of the fungus, although present in the gels, are not visible.
This is clearly illustrated by comparing the results of Chang et al. (1962), and of Williams and Tatum (1966). Work of the first group with *Neurospora crassa* revealed 24 bands with electrophoresis, whereas the second group detected 30 antigenic compounds of the organism by immuno-electrophoresis.
Disc gel electrophoresis appears useful for differentiation of isolates, mainly as a preliminary test and as a supplement to other methods. Wheeler et al. (1971) used this technique to differentiate between cultivars of oats, both resistant or susceptible to *Helminthosporium victoriae*. Isoperoxidase patterns appear to differ and support the conclusion based on field tests.
Electrophoretical patterns can be obtained rapidly and with relatively small amounts of material. Therefore disc gel electrophoresis may be an accessory tool to differentiate isolates of *C. fulvum*. Our data are in agreement with those of Macko et al. (1967) who worked with the soluble proteins of uredospores of *P. coronata* races. However, Gill and Powell (1968) were unable to differentiate between physiological races of *P. fragariae* with this technique. The fact that we found consistent differences between the isolates of *C. fulvum* is probably due to the use of the soluble extracellular proteins from the culture filtrate, purified over Sephadex G-25, whereas most authors cited extracted the whole culture and used these protein preparations as such as sample for their gel electrophoresis.

E Summary

The extracellular soluble proteins of the V_0-fractions of 15 different isolates containing 9 different

physiological races of *Cladosporium fulvum* were obtained from 3 to 4 weeks old shake cultures. The culture filtrates were purified by two gel filtrations over Sephadex G-25; the V_0-fraction was freeze-dried. The obtained preparations were investigated for their electromobility by means of disc gel electrophoresis on polyacrylamide at pH 9.5 at approx. 4 °C.
The various isolates examined appeared to differ consistently in their electrophoretical protein patterns. The length of the incubation period did not influence these patterns. Protein patterns of isolates from different localities but with the same pathogenicity pattern and thus the same genotype for avirulence, do not appear to be identical.

A The resistance of tomato to C. fulvum: 'toxin-induced resistance'

Results described in this dissertation give strong evidence in favour of the hypothesis developed in Chapter II that the natural resistance of certain varieties of tomato plant to certain races of the leaf mould fungus *C. fulvum* is evoked by high molecular weight products of the fungus, which cause a hypersensitive reaction in incompatible tomato plants. Experiments supporting this work have been chiefly described in Chapter IV. However, why the hypersensitive reaction effects cessation of fungal growth remains yet to be elucidated.
Gäumann (1954) brought forward that toxins are involved in some way in most plant diseases, but the decisive importance of toxins in causing the disease symptoms should not be overemphasized. In the host-pathogen combination of oats and *Helminthosporium victoriae* (Samaddar and Scheffer, 1971) it appeared that susceptible oats treated with the pathotoxin victorin isolated from culture filtrates of the fungus *Helminthosporium victoriae* leaked electrolytes and phosphorylated sugars. In contrast, resistant varieties were not affected by this pathotoxin (Luke et al., 1969). This phenomenon occurring also in other diseases caused by Helminthosporium (Chapter I), can be described as a *toxin-induced disease* whereas in the case of apple and *V. inaequalis* as well as of tomato and *C. fulvum* one can speak of *toxin-induced resistance*. In apple the toxin triggers the decompartmentalization and thus the formation of a fungitoxic oxidation product of phloridzin which in its turn kills the penetrating hyphae.
The presence of the fungitoxic compound tomatine in tomato leaf could not be related to the cessation of growth of *C. fulvum* in incompatible tomato, since neither tomatine nor its decomposition or conversion products are fungitoxic or fungistatic to *C. fulvum* (Chapter V).
The only possible explanation so far for the cessation of growth of *C. fulvum* in incompatible combinations is drying out of the invaded tissue as well as of the hyphae as a result of the hypersensitive reaction and the following necrosis. These phenomena may take place on micro-scale.
Some authors (Arneson and Durbin, 1967; Langcake et al., 1972) suggested that tomato pathogens in general owe their ability to infect this plant by decomposing the fungitoxic compound tomatine. This suggestion proves to be untenable. Table V in Chapter V clearly shows that there is no correlation between pathogenicity to tomato and the ability to decompose tomatine.

B Genes for avirulence, and toxins; genes for resistance, and receptors

Any biochemical explanation of resistance must be in accordance with the genetic data.
Table I in Chapter II and Table VII in Chapter IV give the host-parasite relation of tomato plant and *C. fulvum* and the results of leakage experiments. In this specific host-parasite interaction the tomato plant has four genes for resistance. In the literature on the resistance of tomato plant to *C. fulvum* the usual nomenclature for the physiological races (*cf.* Table I, Chapter II) is to nominate the race with the indices on the resistance genes of the tomato variety whose resistance can be broken by that

particular physiological race of the fungus; *i.e.* the race which breaks that resistance of a variety carrying the Cf 1 allele for resistance was nominated as race 1 (*cf.* Hubbeling, 1968). We proposed earlier (*cf.* Van Dijkman and Kaars Sijpesteijn, 1971) that in the host-parasite combination tomato-*C. fulvum* genes for avirulence are involved, and this dissertation has strengthened the evidence for this hypothesis. Hence, the nomenclature used in this dissertation was by proposed genotype for avirulence genes. One might even consider to name the physiological races by their avirulence alleles (A) only analogous to the nomenclature of tomato by dominant Cf alleles. As an example race 1, 2 should than be indicated as race A_3A_4.

Genes for avirulence in *V. inaequalis* causing apple scab have been recognized by Day (in Fincham and Day, 1963). The genetics of resistance in this host-parasite combination shows probably great similarity to that of tomato plant-*C. fulvum* but unfortunately the genotypes of apple with regard to resistance to *V. inaequalis* are still badly understood. Possibly the situation is more complicated than suggested by the work of Boone and Keitt (1957) (*cf.* also Williams and Kuc 1969). Therefore we considered the tomato plant-*C. fulvum* combination as a far more suitable tool to study host-parasite relations where a gene-for-gene system is involved. According to our hypothesis (Chapter II) the four avirulence genes of *C. fulvum* govern the production of 4 different toxins, each evoking leakage of the cell membrane of a tomato leaf cell after a reaction with and/or recognition of a receptor in the membrane of the tomato. The presence of these receptors apparently is controlled by the Cf-genes for resistance. The nature of these fungal toxins has still to be unravelled. They have at least a molecular weight of 1000 (Chapter IV) and may be of protein nature.

Isolates of *C. fulvum* could be differentiated by means of disc gel electrophoresis. Until now in the literature only one case of electrophoretical differentiation within one species has been reported (Macko et al., 1967). Comparison of physiological races of *Phytophthora fragariae* (Gill and Powell, 1968) did not reveal differences in their protein patterns by gel electrophoresis on polyacrylamide. As yet we were unable to find a correlation between the protein patterns in the gels and the products elaborated by the alleles for avirulence of *C. fulvum*.

In view of these reflections and the observations of Müller (1939) and Bond (1938) outlined in Chapter II, breeding for resistance to diseases which have hypersensitivity as a resistance mechanism, may in fact be a selection of varieties in which the receptor sites are present.

The origin of the various physiological races of *C. fulvum* differing in avirulence genes is obscure. Day (1957) succeeded in producing a virulent race by treating an avirulent race with ultraviolet light. This mutation to virulence is a strong indication in favour of avirulence genes.

By introducing tomato varieties resistant to certain races of the fungus inevitably a selection is carried out of races capable to break the newly introduced resistance in the plant. Whether these 'new races' were recently formed by mutation or whether they existed already and were only selected can not be proven. Both possibilities may apply. In such 'new races' apparently an avirulence allele A has mutated into an allele a; consequently the fungus is no longer able to produce a toxic compound which reacts with

the appropriate receptor in the host plant. The hypersensitive reaction is not triggered in that case. Since the production of heterokaryons was still unsuccessful and the existence of a perfect stage of the fungus is still doubtfull, no strict proof can be given that the ability of toxin production is dominant. (Day, 1957).
Four genes for resistance have been introduced into commercial tomatoes. A fifth has already been found in wild Lycopersicon species but has not yet been introduced into the commercial breeding programmes (Hubbeling, 1971).
Peculiar is that the race $A_1A_2A_3A_4$, having all four avirulence alleles, also is outstanding in spore production, growth and protein production: following our hypothesis we assume that other characteristics may have been lost as well due to mutation. The most striking mutations are those concerning the fungal ability to produce the anthraquinone cladofulvine and the loss of ability to produce spores. A similar phenomenon was discovered recently by Day and co-workers (1971): Pathogenic mutants of *Ustilago maydis* were produced by ultraviolet irradiation of cultures of non-pathogenic races of this fungus.
This suggests that the resistance mechanism of corn to *U. maydis* may be similar to that of tomato plants-*C. fulvum*. In the former case the irradiation may then have changed the allele governing the production of the toxin which evokes the hypersensitive reaction and renders the plant resistant. In view of the hypothesis outlined in Chapter II and the results described in Chapter IV the natural resistance of tomato to *C. fulvum* may be explained as follows: one assumes that the incompatibility of a physiological race to certain host varieties depends on the one hand on the production of fungal toxins, which production is governed by avirulence alleles, and on the other hand on the presence in tomatoes of a corresponding receptor in the membrane, which presence is determined by the genes for resistance.

C Considerations on growth in vivo of C. fulvum in incompatible host plants

A limited investigation into the intercellular growth of *C. fulvum* in tomato leaves was undertaken. In view of our theory concerning the hypersensitive reaction as outlined in Chapter II and ou previous publications (Van Dijkman and Kaars Sijpesteijn, 1971, 1972) these observations are of primary importance. It was observed that the chlorosis extends some cells away from the invading hyphae. Thus, material from the fungus must penetrate through these cells and start the decompartmentalization. In this process, chlorophyll is broken down, presumably, by the enzyme chlorophyllase. This observation sustained our view that the hypersensitive reaction is evoked by components excreted by the incompatible fungus. *C. fulvum* does not produce any cellulase or pectinase; this suggested a correlation with the mode of penetration and growth in the leaf. In compatible combinations the fungus seems to grow on the products normally leaking from the cell into the intercellular cavities. It was observed that in compatible combinations the drying out of the leaf started only after the life cycle of the fungus is completed. Parasitism is so well-balanced that the host-leaf cells do not die before spores are produced.

On the other hand with incompatible combinations, local necrosis starts before the mycelium is well-developed.
The velocity of this necrogenic reaction in incompatible plants depends on the Cf allele for resistance involved. Cf 1 and Cf 3 plants react rather slowly resulting in a relatively large chlorotic spot and an extended brown necrotic area. Cf 2 and Cf 4 plants react so quickly that under very optimal conditions only pin-point lesions are observed.
Relating this to our idea of the toxin-induced resistance one might say that the specific toxins formed by races of *C. fulvum* carrying the avirulence alleles A_2 and A_4 evoke a quicker reaction with the membranes of the incompatible host; or have plants with the Cf 2 and Cf 4 gene for resistance more receptor sites, leading to a heavier response upon infection with an incompatible race of the fungus?
As already described in Chapter III and IV the genes Cf 2 and Cf 4 are epistatic over Cf 1 and Cf 3. In the variety V 473 with the genes Cf 1 and Cf 2 combined this epistatic effect can be clearly demonstrated. This can be explained in view of the quicker reaction of the avirulence allele A_2 with the Cf 2 receptor than the reaction A_1 – Cf 1.
When the genes Cf 1 and Cf 3 are both present in one tomato variety the reaction is more like that triggered by Cf 2 or Cf 4. This may be due to a cumulative effect of these genes, i.e. more receptor sites and consequently a quicker reaction when both toxins A_1 and A_3 are involved. However, also when only one of these toxins is presumably present the reaction is like evoked by the genes Cf 2 or Cf 4.

D Semi-permeability and the hypersensitive reaction

The mechanism of leakage is not yet understood. Loss of permeability is frequently reported in plant diseases. Raa (1968b) observed wilting of apple leaves when the leaves were placed on dialyzed culture filtrates of an incompatible race of *V. inaequalis*. Permeability changes are also observed in tulips infected with *Botrytis tulipae* (Schroeder and Schönbeck, 1970).
Inoculation of apple leaf tissue in vitro with an incompatible race of *V. inaequalis* in vivo evoking a hypersensitive reaction did increase the leakage of electrolytes from apple leaf tissues in comparison with an inoculated susceptible leaf (Pellizzari *et al.*, 1970).
Resistant potato cells were damaged almost immediately after penetration by an incompatible race of *P. infestans* (Tomiyama, 1971). Some cells at a distance from the hyphae died within a few minutes after fungal penetration. This is the usual hypersensitive reaction of this incompatible host-parasite combination. In a compatible combination the hypersensitive reaction fails to take place and presumably little leakage of cell components occurs. The symptoms described by Tomiyama and co-workers (1971) are similar to those with tomato and *C. fulvum*.
No leakage experiments were performed on the potato – *P. infestans* combination, but we may expect that the biochemical mechanism of natural resistance is very similar to that described for tomato leaf mould.
Apparently in the hypersensitivity of potato to *P. infestans* as well as in that of tomato to *C. fulvum*,

loss of semi-permeability by the plasma membrane is the primary disease determinant. Vital cellular constituents leak, cells collapse and small lesions are formed within a short distance of the pathogen to bring about the decompartmentalization of the host cells. The tomato leaf mould *C. fulvum* enters the stomata of many non-host plants (Müller, 1939), but in unsuitable ones, the mycelium will die within a short time, presumably from starvation. The hyphae appear to have no effect on the cell membranes. Bond (1938) observed that in some non-host plants a few cells around the penetrating hyphae are killed and produce a local necrotic patch beyond which the fungus does not spread.
In some host-parasite combinations, such as in many rust and mildew infections of resistant varieties of cereals and other plants, the failure of development of the infecting mycelium is ascribed to hypersensitivity of the host tissues which are killed by the close intimacy of fungus and host cells. Of course, these obligate pathogens do not survive when the cells are damaged. It seems attractive to suppose that the resistance mechanisms of these host-parasite combinations are very similar to that described above for tomato leaf mould, depending also on toxin-induced resistance, the toxin being of fungal origin. Thus quite generally a resistance reaction based on hypersensitivity can only occur when the fungus grows intercellularly in incompatible combinations. Should growth be intracellular then the cells are already destroyed and a resistance mechanism based on a hypersensitive reaction can not take place.
In the plant diseases under consideration the resistance mechanism depends on loss of semi-permeability, followed by a decompartmentalization of the intact host cell structure. Available evidence leads to the assumption that in these cases disturbance of the semi-permeability is the primary determinant in the hypersensitive reaction.

E Biochemical tests for assessment of host resistance and differentiation of isolates of C. fulvum

Since a gene-for-gene relationship exists between tomato plants and races of *C. fulvum*, screening for resistance of plants should be possible with the use of our bioassay described in Chapter IV. The high molecular weight fraction produced by selected fungal races upon infiltration into the tomato tissue causes leakage of cellular components.
In screening oat seedlings for resistance to *H. victoriae* practical use has been made of host-specific toxins by exposing them to the toxin of this pathogen (Wheeler and Luke, 1955; Luke, Wheeler and Wallace, 1960).
In Chapter VI the electrophoretic differentiation of different isolates of *C. fulvum* has been described. There are distinct and reproducible differences in the electrophoretical patterns, but on the basis of these preliminary experiments it is not yet possible to judge if a practical application can be elaborated. Determination of the physiological races on the differential host is rather circumstantial and a rapid method for identifying physiological races should be of practical value.
With the aid of modern techniques, such as electron-microscopical autoradiography, tissue culture of the host to refine the bioassay, and quantitative gel electrophoresis much more knowledge

can be acquired about the biochemical background of resistance and susceptibility, necessary for our understanding how Nature accomplishes its natural resistance to plant disease. Such knowledge should be of primary importance for the chemotherapy of plant diseases in future.

Summary

This thesis deals with biochemical investigations on the natural resistance of tomato plants, *Lycopersicon esculentum*, to the leaf mould fungus *Cladosporium fulvum* Cooke.

Chapter I presents a general discussion on natural resistance mechanisms of plants to fungal attack. Emphasis is placed upon the hypersensitive reaction and the gene-for-gene relationship in the resistance of plants towards fungi. Some other resistance mechanisms are briefly discussed.

Chapter II describes the leaf mould disease of tomato caused by *C. fulvum*. The host-parasite relation tomato-*C. fulvum* is a so-called gene-for-gene relationship. By crossing with wild Lycopersicon species until now four resistance genes have been introduced into commercial tomato varieties. Several physiological races of the fungus have been recognized and four independent factors determining pathogenicity were reported (Table I, Chapter II). The disease phenomena described in the literature are surveyed. A hypothesis for the biochemical explanation of the natural resistance of tomato to *C. fulvum* is forwarded postulating that avirulence alleles of the fungus control the excretion of specific toxins which cause a hypersensitive resistance reaction in incompatible tomato plants carrying the corresponding epistatic allele.

Chapter III deals with some studies on growth in vitro and in vivo of *C. fulvum*. The nutritional requirements of *C. fulvum* were investigated. Glucose, saccharose and fructose proved good carbon sources, whereas almost no growth was observed on starch, cellulose and pectin. As a protein-free nitrogen source casamino acids meet the requirements best. No cellulase or pectinase activity was observed in the culture filtrates; apparently the fungus does not need these enzymes for growth in the plant. Inoculation experiments were conducted with the different tomato varieties possessing the various resistance genes. The results are recorded on colour plates.

To check our hypothesis model experiments were carried with the tomato varieties Moneymaker (cf), Leaf Mould Resister No. 1 (Cf 1), Vetomold (Cf 2), V 121 (Cf 3), Purdue 135 (Cf 4), V 473 (Cf 1 Cf 2) and Vagabond (Cf 2 Cf 4), and with various physiological races of *C. fulvum*. In these model experiments, reported in Chapter IV, tissues of tomato plants, containing different genotypes for resistance were exposed to excretion products of different physiological races of *C. fulvum* in an attempt to simulate the in vivo hypersensitive process. A strong correlation was observed between the leakage of ^{32}P labelled compounds from tomato leaf tissue following infiltration with high molecular weight fractions of excretions of *C. fulvum* races, and the occurrence in vivo of the hypersensitive reaction of the host plant to the several fungal races. The toxin-containing high molecular weight fraction of the fungal excretion products was obtained by Sephadex G-25 gel filtration of the culture filtrates.

These observations fully support our hypothesis that the gene-for-gene relation existing between tomato and *C. fulvum* is based on the interaction of fungal excretion products with specific receptors in the host plasma membrane. The synthesis of the active fungal compounds is supposed to be controlled by four avirulence genes (A_1, A_2, A_3 and A_4) whereas the four genes for resistance (Cf 1, Cf 2, Cf 3 and Cf 4) are thought to control the presence of specific receptor sites for these compounds in the

plasma membrane.
The cause of the cessation of fungal growth in incompatible plants after the hypersensitive reaction has taken place, is not yet clear (Chapter V). The possible significance of tomatine or its conversion products in the natural resistance of tomato to *C. fulvum* was investigated. It appeared that neither can be held responsible for the cessation of fungal growth in incompatible combinations of tomato and *C. fulvum*. Although being fungitoxic to many fungi tomatine was ineffective towards this fungus. So far the most plausible explanation for the cessation of fungal growth is drying out of the infected area by loss of semi-permeability. The fungitoxicity of tomatine for a number of other fungal species was assessed. But among those examined no correlation was found between sensitivity to tomatine and non-pathogenicity to tomato. The proposed correlation between the ability of a fungus to hydrolyze tomatine and its pathogenicity to tomato, as proposed in the literature, could not be confirmed.
Some properties of the high molecular weight fractions obtained from the culture filtrates of the various *C. fulvum* races by Sephadex gel filtration are described in Chapters IV and VI. The protein content of the freezedried fractions is rather high (27–60%, w/v). The electromobility of the V_0-fraction was estimated by disc polyacrylamide gel electrophoresis and the protein patterns were characterized. The Sephadex fraction of the various physiological races of *C. fulvum* used revealed different protein patterns. However, also protein patterns of different isolates with the same genotype for avirulence genes were not identical. Cultures of the same isolate grown for different periods of time reveal the same electrophoretical pattern.

The loss in semipermeability in the hypersensitive reaction is considered as the primary determinant factor in this particular resistance mechanism. Attention was given to possible practical application of this study on plant breeding.

References

Albersheim, P. and Anderson, A. J., Proteins from plant cell walls inhibit polygalacturonases secreted by plant pathogens, *Proc. Natl. Acad. Sci.*, 68 (1971) 1815.
Allen, E. H. and Kuc, J., α-Solanine and α-chaconine as fungitoxic compounds in extracts of Irish potato tubers, *Phytopathology*, 58 (1968) 776.
Arneson, P. A. and Durbin, R. P., Hydrolysis of tomatine by *Septoria lycopersici:* a detoxification mechanism, *Phytopathology*, 57 (1967) 1358.

Bailey, D. L., The development through breeding of greenhouse tomato varieties resistant to leaf mould, *Ontario Hort. Expt. Sta. Rep.*, 1945–6 (1947) 56.
Bailey, D. L., Studies in racial trends and constancy in *Cladosporium fulvum*, *Can. J. Res.* (C), 28 (1950) 535.
Bailey, J. H. and Deverall, B. J., Formation and activity of phaseollin in the interaction between bean hypocotyls *(Phaseolus vulgaris)* and physiological race of *Colletotrichum lindemuthianum*, *Physiol. Plant Pathol.*, 1 (1971) 435.
Bailey, D. L. and Kerr, E. A., *Cladosporium fulvum* race 10 and resistance to it in tomato, *Can. J. Botany*, 42 (1964) 1555.
Bailey, D. L. and Langford, A. N., The development through breeding of greenhouse tomato varieties resistant to leaf mold, *Ontario Hortic. Exptl. Sta. Rep.*, 1945/46, p. 56.
Bailey, D. L. and Lowther, R. L., Studies in the nature of resistance in tomato to *Cladosporium fulvum*, *Can. J. Botany*, 40 (1962) 1095.
Barton, D. W., Butler, L., Jenkins, J. A., Rich, C. M. and Young, P. A., Rules for nomenclature in tomato genetics including a list of known genes, *J. Heredity*, 46 (1955) 22.
Beijersbergen, J. C. M., α-Methyleen- γ-butyrolacton uit tulpen, Ph. D. Thesis, State University Leiden, The Netherlands, 1969.
Bennick, A., Detection of persulfate in acrylamide gels, *Anal. Biochem.*, 26 (1968) 453.
Black, W. B., Mastenbroek, C., Mills, W. R. and Peterson, L. C., A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivatives, *Eyphytica*, 2 (1953) 173.
Bond, T. E. T., Infection experiments with *Cladosporium fulvum* Cooke and related species, *Ann. appl. Biol.*, 25 (1938) 277.
Boone, D. M. and Keitt, G. W., *Venturia inaequalis* (Cke) Wint. XII. Genes controlling pathogenicity of wild-type lines, *Phytopathology*, 47 (1957) 403.
Butler, E. J. and Jones, S. G., Plant pathology, Macmillan, London, 1949, p. 672.

Chang, L. O., SRB, A. M. and Steward, F. C., Electrophoretic separations of the soluble proteins of Neurospora, *Nature*, 193 (1962) 756.
Child, J. J., Défago, G. and Haskins, R. H., The effect of cholesterol and polyene antibiotics on the permeability of the protoplasmic membrane of Pythium PRL 2142, *Can. J. Microbiol.*, 23 (1969) 599.
Chrambach, A., Reisfeld, R. A., Wijckoff, M. and Zaccari, J., A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis, *Anal. Biochem.*, 20 (1967) 150.
Ciferro, R., A few critical Italian fungi, *Atti Inst. Bot. Pavia*, Ser. 5, 10 (1954) 237.
Clare, B. G., Starch-gel electrophoresis of proteins as an aid in identifying fungi, *Nature*, 200 (1963) 803.
Cooke, M. C., In: Fungoid pests of cultivated plants, 1906, p. 95.
Cruickshank, I. A. M., Phytoalexins, *Ann. Rev. Phytopathology*, 43 (1963) 351.

Davis, B. J., Disc electrophoresis II. Method and application to human serum proteins, *Ann. N. Y. Acad. Sci.*, 121 (1964) 404.
Day, P. R., Physiologic specialization of *Cladosporium fulvum* in England and Wales, *Plant Pathol.*, 3 (1954) 35.
Day, P. R., Mutants of *Cladosporium fulvum*, *Ann. Rep. John Innes Hort. Inst.*, 1956, p. 15.
Day, P. R., Mutations to virulence in *Cladosporium fulvum*, *Nature*, 179 (1957) 1141.
Day, P. R., Plant disease resistance, *Sci. Prog.*, Oxford, 56 (1968) 357.
Day, P. R., Anagnostakis, S. L. and Puhalla, J. E., Pathogenicity resulting from mutation at the b locus of *Ustilago maydis*, *Proc. Natl. Acad. Sci.*, 68 (1971) 533.
Dekhuijzen, H. M., Transformation in plants of sodium dimethyldithiocarbamate into other fungitoxic compounds, *Nature*, 191 (1961) 198.

De Vries, A., Contribution to the knowledge of the genus Cladosporium Link, Ph. D. Thesis, State University Utrecht, The Netherlands, 1952.
Durbin, R. D., Comparative gel electrophoretic investigation of the protein patterns of Septoria species, *Nature*, 210 (1966) 1186.
Durbin, R. D. and Uchytil, T. F., Purification and properties of a fungal β-glucosidase acting on α-tomatine, *Biochim. Biophys. Acta*, 191 (1969) 176.

Ferenczy, L. and Kevei, F., Data on the mode of action of some antifungal steroidal glycoalkaloids, Wirkungsmechanismen von Fungiziden, Antibiotika und Cytostatika, Mai 1966, Reinhardsbrunn, DDR.
Fincham, J. R. S. and Day, P. R., Fungal genetics, Blackwell Scient. Publ., Oxford, Great Britain, 1963, p. 240–248.
Flor, H. H., Inheritance of pathogenicity in *Melampsora lini*, *Phytopathology*, 32 (1942) 653.
Flor, H. H., The complementary genetic systems in flax and flaxrust, *Advanced Genetics*, 8 (1956) 29.
Fontaine, T. P., Irving, G. W., Ma, R. M., Poole, J. B. and Dootlittle, S. P., Isolation and partial characterization of crystalline tomatine, an antibiotic agent from tomato plants, *Arch. Biochem. Biophys.*, 18 (1948) 467.

Gäumann, E., Toxins in plant disease, *Endeavour*, 13 (1954) 198.
Ghabrial, S. A. and Pirone, T. P., Effects of mineral nutrition on host reaction to *Cladosporium fulvum*, *Phytopathology*, 54 (1966) 493.
Gill, H. S. and Powell, D., Differentiation of three species of Phytophthora by polyacrylamide gel electrophoresis, *Phytopathology*, 57 (1963) 812.
Gill, H. S. and Powell, D., Polyacrylamide gel (disc) electrophoresis of physiologic races A 1 to A 8 of *Phytophthora fragariae*, *Phytopathology*, 58 (1968) 722.
Guba, E. F., Red forcing tomatoes immune to Cladosporium leaf mold, *Mass. Agr. Expt. Sta. Bull.*, (1960) 519.
Guba, E. F. and Rackemann, F. M., Species of Cladosporium on tomato and the allergic response in man as an aid to their identification, *Mycologia*, 30 (1938) 625.

Hall, R., Zentmeyer, G. A. and Erwin, D. C., Approach to taxonomy of Phytophthora through acrylamide gel electrophoresis of proteins, *Phytopathology*, 59 (1970) 770.
Hildebrand, D. C. and Schroth, M. N., Antibiotic activity of pear leaves against *Erwinia amylovora* and its relation to β-glucosidase, *Phytopathology*, 54 (1964) 59.
Hubbeling, N., Identificatie van nieuwe fysio's van *Cladosporium fulvum* en selectie op resistentie daartegen bij de tomaat, *Meded. Rijksfaculteit Landbouww. Gent*, 31 (1966) 925.
Hubbeling, N., Attack of hitherto resistant tomato varieties by a new race of *Cladosporium fulvum* and resistance against it, *Meded. Rijksfaculteit Landbouww. Gent*, 33 (1968) 1011.
Hubbeling, N., Determination trouble with new races of *Cladosporium fulvum* Cooke, *Meded. Rijksfaculteit Landbouww. Gent*, 36 (1971) 300.
Hijwegen, T., Lignification, a possible mechanism of active resistance against pathogens, *Neth. J. Plant Pathol.*, 69 (1963) 314.

Irving, G. W., The significance of tomatine in plant and animal diseases, *J. Wash. Acad. Sci.*, 37 (1947) 293.

Jarvis, W. R., The infection of strawberry and raspberry fruits by *Botrytis cinerea* Fr., *Ann. Appl. Biol.*, 50 (1962) 569.
Jensen, W. A., Botanical histochemistry, Freeman and Comp, London, 1962, p. 202.
Joder, O. C. and Scheffer, R. P., Rôle of toxin in early interactions of *Helminthosporium victoreae* with susceptible and resistant oat tissue, *Phytopathology*, 59 (1969) 1954.

Kaars Sijpesteijn, A., Aspects of natural disease resistance, *Meded. Rijksfaculteit Landbouww. Gent*, 34 (1969) 379.
Kaars Sijpesteijn, A. and Van Dijkman, A., A biochemical basis for the defence reaction of tomato to *Cladosporium fulvum*, *Acta Phytopathol. Acad. Sci. Hung.*, 6 (1971) 79.
Kaars Sijpesteijn, A. and Van Dijkman, A., The host-parasite interactions in resistance of tomatoes to Cladosporium, Third Long Ashton Symposium 1971, University of Bristol, Bristol (U.K.), Fungal pathogenicity and the plants response, 1972, p. 000.
Kelly, F. H. C., Practical mathematics for chemists, Butterworths, London, 1963.
Kern, H., Über die Beziehungen zwischen dem Alkaloidgehalt verschiedener Tomatensorten und ihrer Resistenz gegen *Fusarium lycopersici*, *Phytopathol. Z.*, 19 (1952) 351.

Kerr, E. A. and Bailey, D. L., Resistance to *Cladosporium fulvum* Cooke obtained from wild species of tomato, *Can. J. Botany*, 42 (1964) 1541.
Kooistra, E., Recent experiences of breeding leaf mould resistant tomatoes, *Euphytica*, 13 (1964) 103.
Kuc, J., Resistance of plants to infectious agents, *Ann. Rev. Microbiol.*, 20 (1966) 337.
Kühn, R., Löw, I. and Gäuhe, A., Über das Alkaloid-Glykosid von *Lycopersicum esculentum var. pruniforme* und seine Wirkung auf die Larven des Kartoffelkäfers, *Chem. Ber.*, 83 (1950) 448.

Langcake, P., Drysdale, R. B. and Smith, H., Post-infectional production of an inhibition of *Fusarium oxysporum f. lycopersici* by tomato plants, *Physiol. Plant Pathol.*, 2 (1972) 17.
Langford, A. N., The parasitism of *Cladosporium fulvum* Cooke and the genetics of resistance to it, *Can. J. Res. (C)*, 15 (1937) 108.
Loening, O. E., The fractionation of high-molecular weight RNA by polyacrylamide gel electrophoresis, *Biochem. J.*, 102 (1967) 251.
Loeschke, V. and Butin, H., Über den biologischen und chemischen Nachweis fungistatischer Stoffe in der Rinde verschiedener Pappelsorten, *Meded. Landbouwhogeschool Opzoekstation Staat Gent*, 26 (1961) 1548.
Lowther, R. L., Further studies on the physiology of *Cladosporium fulvum* Cke. and resistance in tomato, *Can. J. Botany*, 42 (1964) 1365.
Luke, H. H. and Wheeler, H. E., Toxin production by *Helminthosporium victoriae*, *Phytopathology*, 45 (1955) 453.
Luke, H. H., Wheeler, H. E. and Wallace, A. T., Victoria-type resistance to crown rust separated from susceptibility to Helminthosporium blight in oats, *Phytopathology*, 50 (1960) 205.
Luke, H. H., Freeman, T. E., Garrard, L. A. and Humphreys, T. E., Leakage of phosphorylated sugars from oat tissue treated with victorine, *Phytopathology*, 59 (1969) 1002.

Macko, V. A., Novacky, A. and Stahmann, M. A., Protein patterns from uredospores of *Puccinia graminis var. tritici*, *Phytopathol. Z.*, 58 (1967) 122.
Malcolmson, J. F. and Black,W., New genes in *Solanum demissum* Lindl. and their complementary races of *Phytophthora infestans* (Mont.) de Bary, *Euphytica*, 15 (1966) 199.
Maurer, H. R., Disk-Elektrophorese, W. de Gruijter and Co., Berlin, West-Germany, 1968, p. 64.
Miller, J. M. and Koeppe, D. E., Southern corn leaf blight: susceptible and resistant mitochondria, *Science*, 173 (1971) 67.
Müller, K. O., Hypersensitivity in plant diseases, *Naturwissenschaften*, 27 (1939) 765.
Müller, K. O., Hypersensitivity. In: J. G. Horsfall and A. E. Dimond, Plant Pathology, Academic Press, New York, N.Y. (U.S.A.), Vol. 1, 1959, p. 469–519.
Müller, K. O. and Börger, H., Experimentelle Untersuchungen über die Phytophthora-Resistenz der Kartoffel, *Arb. Bid. Bund. Inst. Land-u. Forstw. Berlin*, 23 (1940) Heft 2, p. 189.

Nakamura, M. and Ishibashi, K., On the new antibiotic 'ophiobolin' produced by *Ophiobolus miyabeanus*, *J. Agr. Chem. Soc. Japan*, 32 (1958) 732.
Noveroske, R. L., Kuc, J. and Williams, E. B., Oxidation of phloridzin and phloretin related to resistance of Malus to *Venturia inaequalis*, *Phytopathology*, 54 (1964) 92.

Ornstein, L., The disc electrophoresis. I. Background and theory, *Ann. N. Y. Acad. Sci.*, 121 (1964) 321.
Overeem, J. C., Oxidative coupling of alkyl-substituted catechols with phloroacylophenones, *Recl. Trav. Chim. Pays-Bas*, 88 (1969) 851.

Pellizzari, E. D., Kuc, J. and Williams, E. B., The hypersensitive reaction in Malus species: Changes in the leakage of electrolytes from apple leaves after inoculation with *Venturia inaequalis*, *Phytopathology*, 60 (1970) 373.
Persiel, F., Resistenz und Resistenzzüchtung gegen die Braunfleckenkrankheit, *Cladosporium fulvum* Cooke, bei Tomaten, *Z. Pflanzenzüchtung*, 57 (1967) 325.
Person, C., Gene-for-gene relationships in host-parasite systems, *Can. J. Botany*, 37 (1959) 1101.
Person, C., Samborski, D. J. and Rohringer, R., The gene-for-gene concept, *Nature*, 194 (1962) 561.
Pluijgers, C. W. and Kaars Sijpesteijn, A., The antifungal activity of thiosemicarbazides in vitro and their systemic activity against cucumber scab *Cladosporium cucumerinum*, *Ann. appl. Biol.*, 57 (1966) 465.

Quadt, F., Untersuchungen über erbliche Nekrose und ihre Beziehungen zur Resistenz, *der Züchter,* 23 (1953) 223.

Raa, J., Polyphenols and natural disease resistance of apple leaves against *Venturia inaequalis, Neth. J. Plant Pathol.,* 74 (1968,a) 37.
Raa, J., Natural resistance of apple plants to *Venturia inaequalis;* a biochemical study of its mechanism, Ph. D. Thesis, State University Utrecht, The Netherlands, 1968,b.
Raa, J. and Kaars Sijpesteijn, A., A biochemical mechanism of natural resistance of apple to *Venturia inaequalis. Neth. J. Plant Pathol.,* 74 (1968) 229.
Raa, J. and Overeem, J. C., Transformation reactions of phloridzin in the presence of apple leaf enzymes, *Phytochemistry,* 7 (1968) 721.
Rawlins, T. E. and Takashashi, W. N., Techniques of plant histochemistry and virology, National Press, Millbrae, Calif. U.S.A., 1952.
Rohringer, R. and Samborski, D. J., Aromatic compounds in the host-parasite interaction, *Ann. Rev. Phytopathol.,* 5 (1967) 77.

Samaddar, K. R. and Scheffer, R. P., Effects of the specific toxin in *Helminthosporium victoriae* on host cell membranes, *Plant Physiol.,* 43 (1968) 21.
Samaddar, K. R. and Scheffer, R. P., Early effect of *Helminthosporium victoriae* toxin on plasma membranes and counteraction by chemical treatments, *Physiol. Plant Pathol.,* 1 (1971) 319.
Sato, Y., Katz, A. and Mosettig, E., Degradation of tomatidine, *J. Am. Chem. Soc.,* 73 (1951) 880.
Scheffer, R. P. and Ullstrup, A. J., A host-specific toxic metabolite from *Helminthosporium carbonum, Phytopathology,* 55 (1965) 1037.
Schroeder, C. and Schönbeck, F., Permeabilitätsänderungen nach Botrytis Befall und ihr Einfluss auf die Freisetzung von Hemmstoffen in Tulpen, *Phytopathol. Z.,* 69 (1970) 83.
Shipton, W. A. and Fleischmann, G., Disc electrophoresis of proteins from uredospores of races of *Puccinia coronata f. sp. avenae, Phytopathology,* 59 (1969) 883.
Smith, P. M., Last, F. T., Kempton, R. J. and Gisborne, J. H., Tomato leaf mould: its assessment and effects on yield, *Ann. appl. Biol.,* 63 (1969) 19.
Stahmann, M. A., Clare, B. and Woodbury, W., Increased disease resistance and enzyme activity induced by ethylene production by black rot infected sweet potato tissue, *Plant Physiol.,* 41 (1966) 1505.
Stavely, J. R. and Hanson, E. W., Electrophoretic comparison of resistant and susceptible *Trifolium pratense,* non inoculated and inoculated with *Erysiphe polygoni, Phytopathology,* 57 (1967) 482.
Stipes, R. J., Disc electrophoresis of mycelial proteins from Ceratocystis species, *Phytopathology,* 57 (1967) 833.

Termohlen, G. P., De fysiologische specialisatie van *Cladosporium fulvum* en het kweken van tegen de bladvlekkenziekte resistente tomatenrassen, *Tijdschrift Plantenziekte,* 66 (1960) 314.
Thomson, R. H., Natural occurring quinones, 2nd ed., Academic Press, London, 1971, p. 414.
Tomiyama, K., Further observations on the time requirement for hypersensitive cell death of potatoes infected with *Phytophthora infestans* and its relation to metabolic activity, *Phytopathol. Z.,* 58 (1967) 367.
Tomiyama, K., in: S. Akai and S. Ouchi, 'Morphological and related biochemical events in host-parasite interaction', Cytological and biochemical studies of the hypersensitive reaction of potato cells to *Phytophthora infestans,* Phytopathological Soc. of Japan, Tokyo, 1971, p. 387.
Tomiyama, K., Sakai, R., Sakuma, T. and Ishizaka, N., The rôle of polyphenols in the defense reaction in plants induced by infection. In: C. J. Mirocha and I. Uritani, The dynamic rôle of molecular constituents in plant-parasite interaction, Bruce Publ. Co., St. Paul, Minn. (U.S.A.), 1967, p. 165–182.
Trudgill, D. L. and Carpenter, J. M., Disk electrophoresis of proteins of Heterodera species and pathotypes of *Heterodera rostochiensis, Ann. appl. Biol.,* 69 (1971) 35.
Tschesche, R., Kämmerer, F. J. and Wulff, G., Über die Struktur der antibiotische aktiven Substanzen der Tulpe *(Tulipa gesneriana* L.*), Chem. Ber.,* 102 (1969) 2057.
Turner, E. M. C., An enzymic basis for pathogenic specificity in *Ophiobolus graminis, J. Expt. Bot.,* 12 (1961) 169.

Van Dijkman, A. and Kaars Sijpesteijn, A., A biochemical mechanism for the gene-for-gene resistance of tomato to *Cladosporium fulvum, Neth. J. Plant Pathol.,* 77 (1971) 14.

Van Dijkman, A. and Kaars Sijpesteijn, A., Membrane leakage in the hypersensitive host reaction of tomato to *Cladosporium fulvum* Cooke, submitted for publication.
Van Loon, L. C. and Van Kammen, A., Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN'. *Phytochemistry*, 7 (1968) 1727.
Van der Plank, J. E., Disease resistance in plants, Academic Press, New York, N.Y. (U.S.A.), 1968.
Verhoeff, K., Spotting of tomato fruits by *Botrytis cinerea*, *Neth. J. Plant Pathol.*, 76 (1970) 219.

Wheeler, H. E. and Luke, H. H., Mass screening for disease resistant mutants in oats, *Science*, 122 (1955) 1229.
Wheeler, H. E. and Luke, H. H., Microbial toxins in plant disease, *Ann. Rev. Microbiol.*, 17 (1963) 223.
Wheeler, H. E., Novacky, A. and Luke, H. H., Isoenzymes of Victoria blight-resistant oat lines selected from susceptible cultivars, *Phytopathology*, 61 (1971) 1147.
Williams, E. B. and Kuc, J., Resistance in Malus to *Venturia inaequalis*, *Ann. Rev. Phytopathol.*, 7 (1969) 223.
Williams, C. A. and Tatum, E. L., Immuno-electrophoresis analysis of cytoplasmic protein of *Neurospora crassa*, *J. Gen. Microbiol.*, 44 (1966) 59.
Wood, R. K. S., Pectic and cellulolytic enzymes in plant disease, *Ann. Rev. Plant Physiol.*, 11 (1960) 299.
Wood, R. K. S., Physiological plant pathology, Botanical Monographs, Blackwell Scientific Publications, Oxford, Great Britain, 1967, p. 491–509.

Samenvatting

Deze dissertatie omvat een biochemisch onderzoek over de natuurlijke resistentie van tomaten, *Lycopersicon esculentum*, tegen de bladvlekkenziekte, veroorzaakt door *Cladosporium fulvum* Cooke.
Hoofdstuk I houdt een algemene discussie in over de natuurlijke resistentie mechanismen van planten tegen schimmel aantastingen. De nadruk wordt gelegd op de overgevoeligheidsreactie en de gen-om-gen relatie tussen de resistentie van planten tegen schimmel aantastingen. Enige andere resistentie mechanismen worden in het kort behandeld.
Hoofdstuk II beschrijft de bladvlekkenziekte van de tomaat veroorzaakt door *C. fulvum*. De plant-parasiet relatie tomaat-*C.fulvum* is een zogenaamde gen-om-gen relatie. Door kruisingen van niet-in-cultuur-zijnde Lycopersicon soorten zijn tot nu toe vier resistentiegenen ingekruisd in commercieel gebruikte rassen. Verscheidene fysiologische rassen van de schimmel zijn tot nu toe geïsoleerd en vier onafhankelijke factoren, die de pathogeniteit zouden bepalen, zijn gevonden. (Tabel I, Hoofdstuk II).
Verder wordt een overzicht gegeven van de ziekte symptomen die in de literatuur zijn beschreven. Aan het eind van dit hoofdstuk wordt een hypothesis geponeerd voor de natuurlijke resistentie van tomatenplanten tegen de bladvlekkenziekte parasiet *C. fulvum*. Deze hypothese houdt in dat de avirulente allelen van de schimmel de productie en uitscheiding regelen van specifieke toxinen die een hypersensitieve resistente reactie bewerkstelligen bij incompatibele combinaties van tomatenplanten die een of meerdere overeenkomstige allelen bezitten.
Hoofdstuk III bevat een verslag over enige onderzoekingen over de groei in vito en in vivo van *Cladosporium fulvum*. De voedingsbehoeften van *C. fulvum* worden onderzocht. Glucose, saccharose en fuctose geven goede groei van de schimmel, terwijl echter nauwelijks enige groei werd waargenomen op een bodem die zetmeel, cellulose of pectine bevatte. Als eiwit-vrije stikstofbron voldeed casamino acids het beste. Geen cellulose of pectinase activiteit werd waargenomen in de cultuurfiltraten; blijkbaar heeft de schimmel deze enzymen niet nodig om intercellulair te groeien.
Inoculatie experimenten werden uitgevoerd met verschillende tomaten rassen, die ieder verschillende resistentie genen bezaten. De resultaten werden vastgelegd op kleurenfoto's.
Om de waarde van deze hypothese te onderzoeken werden modelproeven uitgevoerd met tomaten rassen Moneymaken (cf), Leaf Mould Resister No. 1 (Cf 1), Vetomold (Cf 2), V 121 (Cf 3), Purdue 135 (Cf 4), V 473 (Cf 1 Cf 2) en Vagabond (Cf 2 Cf 4) in combinatie met verschillende fysiologische rassen van *C. fulvum*. In deze modelproeven (zie Hoofdstuk IV) werden weefsels van tomatenplanten, met verschillend genotype voor resistentie, in contact gebracht met uitscheidingsproducten van verschillende fysiologische rassen van *C. fulvum*, in een poging een simulatie te krijgen van de hypersensitieve reactie in vivo. Een sterke correlatie werd gevonden tussen de uitlek van ^{32}P– gemerkte verbindingen uit tomatenblad, na de infiltratie van dit tomatenbladweefsel met de hoogmoleculaire fractie van de uitscheidingsproducten van *C. fulvum* rassen, en het voorkomen van de hypersensitieve reactie in vivo van de waardplant na inoculatie met de verschillende rassen van *C. fulvum*. De fracties die stoffen met een hoog molecuul gewicht bevatten, werden verkregen door de cultuurfiltraten van de schimmel over Sephadex G-25 te filtreren. Deze waarnemingen ondersteunden ten volle onze

hypothese dat de gen-om-gen relatie tussen tomaat en *C. fulvum* gebaseerd is op de interactie van specifieke excretieproducten van de schimmel met specifieke receptors in de celmembraan van de gastheerplant. De synthese van deze actieve schimmel producten wordt verondersteld geregeld te worden door vier avirulente genen (A_1, A_2, A_3 en A_4), terwijl de vier genen voor resistentie (Cf 1, Cf 2, Cf 3 en Cf 4) de aanwezigheid van de specifieke receptoren voor deze stoffen in de celmembraan zouden regelen.
De oorzaak van het stoppen van de schimmelgroei in incompatibele planten, nadat de hypersensitieve reactie heeft plaats gevonden, is nog niet opgehelderd. (Hoofdstuk V). De mogelijke rol van tomatine of omzettingsproducten daarvan in de natuurlijke resistentie van tomaten tegen *C. fulvum* werd onderzocht. Het bleek echter dat geen van beide verantwoordelijk konden zijn voor remming van de schimmelgroei in incompatibele combinatie van plant en schimmel Hoewel tomatine fungitoxisch is voor vele schimmels is de stof dat niet voor *C. fulvum* De meest aannemelijke verklaring tot nu toe voor het remmen van de groei is het uitdrogen van de plaats van binnendringen doordat de semi-permeabiliteit van de omringende cellen verloren gaat. De toxiciteit van tomatine voor een aantal andere schimmelsoorten werd getest. Er werd echter geen correlatie gevonden tussen de schimmels die al of niet gevoelig waren en de pathogeniteit op tomaten. De geopperde correlatie tussen de mogelijkheid van een schimmel om tomatine te hydroliseren en zijn pathogeniteit ten opzichte van tomaten, zoals in de literatuur vermeld wordt, kon niet bevestigd worden.
Enige eigenschappen van de fracties met hoog molecuulgewicht verkregen uit de cultuur filtraten van verschillende fysiologische rassen door middel van gelfiltratie over Sephadex G-25, werden beschreven in de Hoofdstukken IV en V. Het eiwitgehalte van de drooggevroren fracties is tamelijk hoog (27–60%). De mobiliteit in een electrisch veld van de V_o-fracties werd bepaald met disc gel electrophorese op polyacrylamide. De eiwitpatronen werden gekarakteriseerd. De patronen van de verschillende Sephadex fracties van de fysiologische rassen van *C. fulvum* vertoonden verschillen. Echter, ook eiwitpatronen van verschillende isolaties, maar met hetzelfde avirulente genotype, vertoonden verschillen. Cultures van dezelfde isolaties die op verschillende tijden gekweekt waren, gaven hetzelfde electrophoretische patroon.
Het verlies van de semipermeabiliteit bij de resistentie gebaseerd op de hypersensitiviteit wordt beschouwd als de eerste reactie die van fundamenteel belang is. De mogelijke practische toepassingen van de resultaten van dit onderzoek werden besproken.

Curriculum vitae

Op verzoek van de faculteit van de Wiskunde en Natuurwetenschappen.

Antoon van Dijkman werd geboren op 18 januari 1940 te Amsterdam. De middelbare school werd in Haarlem doorlopen en het H.B.S.-B diploma werd behaald op het Coornhert Lyceum in 1958. In hetzelfde jaar werd de studie in de biologie aan de Universiteit van Amsterdam aangevangen. Het kandidaatsexamen werd afgelegd in 1962 en in juni 1965 volgde het doctoraalexamen biologie met als uitgebreid hoofdvak plantenfysiologie en de bijvakken fytopathologie en economie. Vanaf 1 september 1965 tot 1 juli 1972 was de schrijver werkzaam op de Biochemische en Microbiologische Afdeling van het Organisch Chemisch Instituut TNO te Utrecht, alwaar dit proefschrift werd bewerkt. Per 1 juli 1972 trad de auteur in dienst van Gist-Brocades N.V. te Delft, waar hij als produktontwikkelingsleider werkzaam is in de Divisie Industriële Producten.
De schrijver is gehuwd met Drs. E. H. Nieuwenhuijzen, biologe, en is vader van twee zoons.