II

NARCISSUS SMOULDER;
CAUSE, EPIDEMIOLOGY AND HOST RESISTANCE

BY

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The work presented in this thesis is the result of my own investigation and has neither been accepted nor is being submitted for any other degrees.

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ABBREVIATIONS AND CHEMICAL FORMULAE

1. Abbreviations

ARC	Agricultural Research Council
a.u.f.s.	absorbance units full scale (deflection)
BC	*Botrytis cinerea*
BF	*Botrytis fabae*
BN	*Botrytis narcissicola*
b.p.	boiling point
BT	*Botrytis tulipae*
c.	circa
CMI	Commonwealth Mycological Institute
coA	Coenzyme A
conc.	concentrated
cv.	cultivar
diam.	diameter
DMSO	Dimethylsulphoxide
DpNA	Diazotised p-nitroaniline
ESCA	East of Scotland College of Agriculture
ED_{50}	Effective dose (50%) (see text)
fr.wt.	fresh weight
GCRI	Glasshouse Crops Research Institute
GTL	Germ tube length
h	hours
HPLC	High Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HWT	Hot Water Treatment
I	Inhibition
LL	Limited Lesion
M^+	Molecular ion
m/e	mass/charge ratio

min	minute
MS	Mass Spectrum
m.p.	melting point
NL	No Lesion
NMR	Nuclear Magnetic Resonance (spectrum)
NOSCA	North of Scotland College of Agriculture
ODS	Octadecyl sulphate
PA	phytoalexin
PAL	Phenylalanine ammonia lyase
PC	Preparative chromatography (thin layer)
PDA	Potato dextrose agar
RDA	retro-Diels-Alder
SCC	Stirling University Culture Collection
SDW	Sterile distilled water
SEM	Standard error of the mean
sh	shoulder
SHRI	Scottish Horticultural Research Institute
SL	Spreading lesion
sp.	species
SPN	synthetic pod nutrient
TLC	Thin layer chromatography
TS	Total sites (inoculations)
UV	Ultra violet
VT	Virus tested
wk	week
yr	year

2. Chemical formulae

$AlCl_3$	Aluminium chloride
AmOH	Amyl alcohol
$CHCl_3$	Chloroform
Cl_2	Chlorine
Et_2O	Diethyl ether
Et_2OAc	Ethyl acetate
EtOH	Ethanol
$FeCl_3$	Iron(III) chloride
HCl	Hydrochloric acid
HCO_2H	Formic acid
H_2SO_4	Sulphuric acid
KCl	potassium chloride
KOH	Potassium hydroxide
MeCN	Acetonitrile
MeOH	Methanol
Na_2CO_3	Sodium carbonate
NaOCl	Sodium hypochlorite
NH_3	Ammonia
$NH_2OH.HCl$	Hydroxylamine hydrochloride
methanol-d_4	deuterated methanol
pyridine-d_5	deuterated pyridine

ABSTRACT

The cause and epidemiology of narcissus smoulder and the mechanisms by which daffodil bulbs resist infection by Botrytis were investigated.

Isolates of B. narcissicola and B. cinerea were not easily distinguished by conidiophore or conidial morphology but were readily identified by sclerotia produced on potato dextrose agar. They were also distinguished by their pathogenicity to narcissus from mycelial inocula. The majority of isolates collected from field-grown narcissus were B. narcissicola. Following inoculation with conidia in sterile water, both species typically failed to colonise narcissus. However, damaging tissue or adding nutrients allowed B. narcissicola to infect. It was concluded that B. narcissicola is the major cause of smoulder.

Healthy bulbs inoculated with B. narcissicola, or grown in soil containing botrytis sclerotia, developed lesions in the shoot (primary symptoms). Botrytis narcissicola was commonly isolated from the bulb neck of plants with primary symptoms; it is suggested that infected bulbs are the major source of smoulder outbreaks.

Secondary infection by conidia was enhanced by damaging leaves, and open stalk ends left after flower picking were found to provide an important site for infection development. Botrytis narcissicola was isolated from bulb necks when plants with symptoms of secondary infection had died down. The effects of prolonged storage, planting depth and soil type on the production of primaries from infected bulbs are discussed.

Following conidial inoculations, lignification and phytoalexin accumulation were detected in bulb scales. Three out of twelve phytoalexins were identified, as closely related hydroxyflavans. Botrytis narcissicola was found to be as sensitive to the inhibitors as non-pathogenic Botrytis species. From the inability to detect phytoalexins or extensive lignification in or around spreading lesions, it was concluded that pathogenicity of B. narcissicola depends on its ability to suppress the host's resistance mechanisms.

A range of flavonoid compounds related to the hydroxyflavan phytoalexins were assayed in an attempt to define the structural requirements for antifungal activity.

PREFACE

The experimental section of this thesis contains three chapters, I: The cause of smoulder and infection of narcissus by species of Botrytis, II: Smoulder epidemiology, and III: Mechanisms of resistance to infection. The introduction and literature review, materials and methods, and discussion each contain corresponding sections so that, if the reader so wishes, the three experimental chapters may be treated as separate entities.

C O N T E N T S

INTRODUCTION AND LITERATURE REVIEW

SECTION 1

THE CAUSE OF SMOULDER AND THE INFECTION OF NARCISSUS BY SPECIES OF *BOTRYTIS*

1. Cultivation of narcissus in Scotland

The growing of narcissus in Scotland probably started in the late 19th century. Production on a farm scale began in 1913 and by 1939 the area of land under bulbs was *c*. 50ha. Flower bulb production increased after the second World War and expanded again in the 1960s. The area of narcissus, the major Scottish bulb crop, increased from 100ha in 1960 to a peak of 290ha in 1973 (Turner, 1975). The area of narcissus now grown in Scotland, *c*. 200ha, is about 5% of the total UK growing area, 3,600ha.

Most of the narcissus crop in Scotland is grown in the north-east, in Angus and Kincardineshire. The bulbs are grown in ridges and are usually left in the ground for two years, although stocks for flower production, a minority, may be left down for 5 years or more. Scottish bulb growers have applied their experience of growing certified seed potatoes to the production of healthy bulb stocks. Roguing of diseased, particularly smoulder and virus-infected plants, is carried out routinely. A narcissus bulb certification scheme was introduced by the Department of Agriculture and Fisheries for Scotland in 1969 (Ebbels, 1979). To obtain a certificate the growing crop had to be 97% free from plants with obvious virus symptoms, eelworm infection, smoulder, and other pests and diseases. A higher certificate was

introduced in 1975 with a tolerance level of only 1% severe virus symptoms and in 1977 a bulb growers nuclear stock association was formed to promote the development, multiplication and distribution of healthy, virus-tested (VT) bulbs.

Work started on the production of VT clones of narcissus at the Glasshouse Crops Research Institute (GCRI) Littlehampton, in 1963, beginning with meristem tip culture of cv. Grand Soleil d'Or (Stone, 1973). Results were promising - virus free bulbs grew more vigorously than infected ones and gave more and larger flowers with a better colour (Stone, 1973). This work was extended to other cultivars both at GCRI and at the Scottish Horticultural Research Institute (SHRI). Rapid multiplication of VT narcissus clones to commercial quantities was made possible by the application of twin-scaling propagation to narcissus (Everett, 1954; Anon, 1973; Alkema, 1975; Stone, Brunt and Hollings, 1975; Hanks and Rees, 1978). The second cycles of twin-scaling in Scotland are now carried out by the North and East of Scotland Colleges of Agriculture.

Smoulder is common wherever narcissus is grown, but opinions differ on its economic importance (Moore, 1979). Millar (1975) reported similar bulb yields from infected and healthy stocks after one and two years in the ground. In northern Scotland however, Gray and Shiel (1975) reported that smoulder may reduce both bulb yield and flower size. With the imminent dispersal of disease-free bulbs to re-stock Scottish plantings, the need to further investigate narcissus smoulder and devise rational methods of control has become more urgent.

2. Narcissus smoulder disease

A. History

A botrytis disease of narcissus was first described by Klebahn in 1907. When bulbs bearing sclerotia were kept damp, conidiophores characteristic of Botrytis developed on leaves and bulb scales. He demonstrated the pathogenecity of the fungus towards narcissus by infecting leaves from sclerotia placed over the bulb nose at planting. Although unable to decide whether the pathogen was identical with other species of Botrytis he suggested the name B.narcissicola. In Holland, Westerdijk (1911, 1916) described a narcissus disease known as 'smeul' (smoulder) caused by an unnamed species of Botrytis. She noted the similarity of the disease to tulip fire, caused by Botrytis parasitica (syn. Botrytis tulipae). In 1928 she and van Beyma gave morphological and cultural descriptions of the fungus, which they then recognised as B.narcissicola, the species described by Klebahn. In England, Dowson (1924) gave a short account of a sclerotial disease of narcissus on bulbs imported from Holland and later (1926) identified the fungus as B.narcissicola. The disease was reported in Scotland in 1942 by Dennis and Foister. Moore (1939) reviewed narcissus smoulder in his book on bulb diseases and this publication has recently (1979) been revised.

B. The Pathogen

In the original account of smoulder by Klebahn, the conidiophores of B.narcissicola were described as up to 1mm tall, grey brown in colour below and lighter above, some unbranched and others, either near the tip or lower down, alternately branched. The conidia were described as oval,

a little pointed towards the lower end, smooth, light brown and measuring 10-12 x 6-7µm. Westerdijk (1916) stated that the conidia of the smoulder pathogen were larger than those of B. cinerea and later (1928) gave the dimensions of B. narcissicola conidia as 8-16 x 7.5-12µm (average: 12.3 x 9.5µm). Klebahn noted that sclerotia on naturally infected bulbs differed in size and following artificial infection recorded sclerotia up to 4 x 1-2mm. Westerdijk and van Beyma (1928) recorded B. narcissicola sclerotia grown on pieces of potato as 0.5mm in diameter. Dowson (1924, 1928a) observed that B. narcissicola sclerotia in vivo were larger and flatter than those of B. tulipae and similar to B. allii, measuring 1/16 - 1/8" (1.6 - 3.2mm) across. Gregory (1941) provided evidence of a connection between B. narcissicola Kleb. and the perfect species Sclerotinia. He proposed the name S. narcissicola and fully described the apothecial stage. Cultures grown on PDA from single ascospores and single conidia both produced smooth, black, rounded sclerotia, 10-15mm in diameter, evenly distributed over the surface of the medium. Whetzel (1945), within a new family sclerotiniaceae, erected a genus Botryotinia composed of those species with Botrytis conidial states. This was subsequently reappraised by Buchwald (1949) who subdivided the genus according to conidial morphology. The perfect state of the smoulder pathogen was hence to be known as Eubotryotinia narcissicola (Gregory) Buchw. Present knowledge of narcissus smoulder indicates that ascospores play little part in the epidemiology, rarely being found in the field, and I shall therefore refer to the pathogen as Botrytis narcissicola.

C. Symptoms

Narcissus smoulder has been recognised by a variety of symptoms in foliage, flowers and bulbs. Westerdijk (1911, 1916) observed shoots already infected at emergence with shrunken leaves covered in botrytis conidiophores. She suggested that the fungus attacks at the leaf base as young shoots come above ground, causing leaves to wither, turn brown and rot; the leaves of infected plants were easily pulled off. In the U.S.A. McWhorter and Weiss (1932) also described narcissus shoots emerging in a blighted and deformed condition. The leaves were crumpled and sometimes failed to separate, brown streaks appeared on the leaf tips or margins and sclerotia developed in these lesions. During wet weather the sclerotia released conidia. Dowson (1926), describing the growth of plants from imported bulbs bearing sclerotia noted that leaves of infected plants were paler than normal, soon turned yellow at the base, and withered upwards. In three instances out of four the diseased bulbs produced no foliage at all.

The degree of damage to narcissus flowers described ranges from brown spotting of petals (Moore, 1939) through blighted shoots (McWhorter and Weiss, 1932) to complete failure of flower production (Westerdijk, 1911). More recent descriptions of smoulder have noted water-soaking of the innermost scale leaf (sheath) in shoots with leaf lesions (Anon., 1970). In observations on smoulder in northern Scotland, Gray (1971) described curving of the first foliage leaf with chlorosis along the inner margin as an early indication of smoulder. According to Gray, the most usual symptom (in Scotland) was a brown lesion, dry or bearing

conidia, at the tip of leaves or on the flower bud or part of the stalk.

In addition to leaf and flower damage the term smoulder has also been used to describe a bulb rot. The experience of Dowson (1928a) was that bulbs bearing sclerotia of botrytis rarely grew and generally rotted away in the ground. Moore (1939) stated that with prolonged storage into the autumn B. narcissicola began to attack the basal plate and bulb scales, producing a yellow-brown tissue rot. Hawker (1940) noted a greater loss to B. narcissicola when bulbs were stored at a cool (4-10°C) or moderate (17°C) rather than a warm (25°C) temperature. B. narcissicola has also been implicated, together with Stagonospora curtsii, Fusarium spp. and Penicillium spp., in narcissus neck rot (P.J. Muller, pers. comm.; Bergman and Nordermeer, 1975; Price, 1978; Millar, 1978; Moore, 1979).

Moore (1939) stated that leaf tips damaged by the wind, particularly during cool wet seasons, could be infected by botrytis spores. He suggested that B. cinerea, rather than B. narcissicola, was mainly responsible for severe damage caused in this manner. Westerdijk and von Beyma (1928) had also reported that B. cinerea was found in leaf tip lesions. Humphreys-Jones (1975) stated that identical smoulder symptoms could be caused by B. narcissicola and B. cinerea.

3. Infection of narcissus by species of Botrytis

A. Host-specificity

(i) Species. Species of Botrytis are important plant pathogens on vegetables, glasshouse crops, forest tree seedlings, small fruits, vines and bulbous monocotyledons

(Jarvis, 1977). Of 25 Botrytis species listed by Hennebert (1973), 14 occurred on host species listed in the monocotyledenous families, Liliaceae, Amaryllidaceae and Iridaceae.

Most Botrytis spp. show a fairly close degree of host-specificity and are accordingly given the host name in the specific epithet. The exception is the plurivorous B. cinerea for which MacFarlane (1968) listed more than 200 hosts. Jarvis (1977) noted that the most host-specific Botrytis spp. occurred on members of the monocotyledon division Corolliferae and the related, dicotyledonous, Ranunculaceae. In interactions of Botrytis spp. with Allium spp. a range of host specificities has been described (Hennebert, 1963). For example, while seven Botrytis spp. occurred on A. cepa, only B. sphaerosperma was pathogenic and confined to A. triquetrum. Single-host specificity is probably shown also by B. globosa, B. spermophila, B. anthophila and B. pelargonii (Jarvis, 1977). Less restricted host-specificity is shown by B. tulipae (known to occur on Lilium regale as well as Tulipa spp.) by B. elliptica and by B. gladiolorum (Jarvis, 1977). Dowson (1928a) reported that B. narcissicola could infect Galanthus as well as narcissus.

(ii) Isolates. Isolates of the same Botrytis sp., particularly B. cinerea, may differ widely in the range of host plants which each can attack (e.g. Paul, 1929). Several workers have erected formae speciales, usually on the basis of pathogenicity tests, which were believed to reflect host-specificity (Jarvis, 1977). Isolates of a single Botrytis sp. collected from one host may also differ

widely in their virulence towards that host; for example, isolates of B. cinerea from lemon (Klotz, Calavan and Zentmeyer, 1946) and isolates of B. fabae from Vicia faba (Deverall, Smith and Makris, 1968). By contrast, Schnellhardt and Heald (1936) recorded an apparent lack of host-specificity for some isolates of B. cinerea collected from a range of host plants.

B. Conditions influencing infection

(i) Nature of inoculum. One factor which strongly influences the ability of Botrytis to infect a particular host is the nature of the inoculum. Natural infections frequently involve conidia, less frequently mycelium and rarely, if at all, ascospores of the corresponding Botryotinia species (Jarvis, 1977). Although conidia are commonly the most abundant type of inoculum, inoculations from mycelium may be the more successful. Jarvis (1962) found that only c. 1% of infections of intact, ripe strawberry and raspberry fruits occurred from conidia, germinating in a persistent drop of water on the fruit surface; all other infections occurred from mycelium in a saprophytic base.

The importance of a saprophytic base was recognised almost a century ago (de Bary, 1886; Brooks, 1908). B. cinerea often first establishes as a saprophyte in senescent flowers and from them infects other tissue, either directly by growth down the peduncle or indirectly if the flower falls onto other tissue. Similarly, dead leaves and other tissues can serve as saprophytic bases (e.g. Brown and Montgomery, 1948; Lipton and Harvey, 1960).

Where fungi have attempted infection, but failed to colonize, a limited lesion may result. For example, Segall (1953) found that onion leaf spotting ('blast') could result from inoculation with conidia of B. allii, B. cinerea, B. tulipae or B. paeoniae. Ainsworth, Oyler and Read (1938) reproduced tomato 'ghost spot', a limited flecking of tomato fruits, by inoculation with B. cinerea and Verhoeff (1970) has successfully isolated B. cinerea from ghost spot lesions. Reasons for fungi failing to progress further through host tissue after limited lesion formation are discussed in Section III.

(ii) Age of host tissue. Plant tissue may change in susceptibility to infection with senescence. Valaskova (1963), for example, found that premature tulip leaf senescence, resulting from potassium and magnesium deficiencies, led to increased infection by B. tulipae. Wilson (1963) found that tomato stems initially resistant to B. cinerea conidia inoculated at a leaf scar became susceptible with increased age. Changes in tissue susceptibility with senescence may be explained in part by changes in nutrient availability. Horsfall and Dimond (1957) suggested that Botrytis species attack tissues with a high sugar content, classifying them as high-sugar pathogens. Grainger (1968) proposed a more general theory which indicated that the susceptibility of a plant to infection was likely to change with major developmental events (e.g. flowering) as well as with age. The failure of active mechanisms of host resistance in dying tissues (Mansfield, 1980) is more probably the major feature responsible for increased susceptibility.

(iii) Wounds. Wounding frequently enhances the ability of B. cinerea to colonise tissue (Jarvis, 1977). Buxton, Last and Nour (1957) and Last (1960) reported that infection by conidia of host-specific Botrytis spp., especially old conidia of low infectivity, was also enhanced if the host plant was wounded. In the field natural wounds may result from wind, wind-blown soil particles, machinery or various biotic agents.

(iv) Nutrients. Exogenous nutrients frequently stimulate infection by B. cinerea conidia; for example, on broad bean (Brown, 1922; Chou, 1972), cabbage (Yoder and Whalen, 1975), onion (Clark and Lorbeer, 1976), stored carrots (Sharman and Heale, 1979) and grapevine (Deramo, 1980). One source of nutrients is the host tissue, solutes moving by exosmosis into infection drops (Brown, 1922). The major components entering infection drops are probably simple reducing sugars and amino acids (Kosuge and Hewitt, 1964).

Other natural sources of nutrients are aphid honeydew (Last, 1960) and pollen (Brown, 1922; Chou and Preece, 1968). Pollen in particular has been noted as an effective stimulant of conidial infections, and of many other weak pathogens, on a range of host plants (Deramo, 1980). The stimulatory factor provided by pollen does not appear to consist of a single, active component but more probably results from a mixture of substances (Chou and Preece, 1968; Warren, 1972; Deramo, 1980). Strange, Majer and Smith (1974) identified choline and betaine at two major components of wheat anthers that stimulate Fusarium graminearum in vitro.

The general effect of an increased nutrient level is to stimulate spore germination, germ tube growth and appressorium formation, leading to increased penetration of inoculated tissues. At the extreme, a fungus on a given host may be transformed from a non-pathogen to a pathogen. For example, B. allii was induced to attack apple fruits by adding nitrogen salts (Vasudeva, 1930; Chona, 1932).

SECTION II

SMOULDER EPIDEMIOLOGY

1. The disease cycle

Although smoulder is a frequent and widely distributed disease, little experimental work has been done in connection with the life cycle of *B. narcissicola* (Moore, 1979). A disease which in many respects appears similar to narcissus smoulder, and on which much work has been undertaken, is tulip fire caused by *B. tulipae*. I shall therefore give a brief account of this disease, with particular reference to fire epidemiology and the life cycle of *B. tulipae*, as this forms a useful parallel for studies on narcissus smoulder.

Tulip fire attacks all parts of the tulip plant, causing a spotting of leaves and flowers and a rot of the bulbs. Experimental work on tulip fire has been carried out by workers in England (Beaumont, Dillon Weston and Wallace, 1936; Price, 1967, 1970 a, b, c; Price, Turquand and Wallis, 1971; Price and Briggs, 1974) and in Holland (Doornik and Bergman, 1971, 1973, 1974, 1975). Their research has shown that the disease is usually introduced into crops by planting contaminated bulbs. Infected shoots, known as primary infectors or primaries, emerge from some of these bulbs. Shoots may also emerge as primaries from bulbs planted in soil infested with *B. tulipae*; such infections are probably important in parks and gardens where bulbs are grown in the same soil in consecutive years. Spores from primary infectors produce secondary spotting on other tulip plants. Most infections remain as limited spots

but some develop into spreading lesions. Sporulation on aggressive lesions causes a further cycle of spotting on leaves, flowers and buds and, given suitable conditions, a fire epidemic can soon develop. The disease is carried over from one season to the next by survival of the fungus either in the soil (as sclerotia) or on the bulb. Infection of previously healthy bulbs can occur by transfer of the fungus from an infected mother bulb to adpressed daughter bulbs, by growth of mycelium down the flower stalk, or from conidia washed down from aerial parts into the bulb necks. An economic level of disease control is now possible using a combination of fungicide treatments (pre-planting bulb dips and foliage sprays) and thorough roguing of diseased plants.

Returning to narcissus, the present knowledge and speculation on smoulder epidemiology may be summarized as follows. In view of the correlations reported between the presence of Botrytis sclerotia in outer bulb scales and the development of infected shoots on planting such bulbs (Dowson, 1926; McWhorter and Weiss, 1932) it is probable that the disease, and more specifically B. narcissicola, is bulb-borne. This proposal is supported by reports that the disease occurs initially in individual plants scattered within a field (Moore, 1939). However, the presence or absence of sclerotia in bulbs is not always a reliable indicator as to which plants will show foliage smoulder at emergence. Indeed, Gray, Shaw and Shiel (1975) and Humphreys-Jones (1975) both found no relation between sclerotia in the outer scales and shoot infection. A partial explanation for conflicting reports may be that

B. cinerea sclerotia, indistinguishable from those of *B. narcissicola*, are also present on bulbs (Moore, 1979).

The location of *B. narcissicola* infections in bulbs, and subsequent routes of fungal development (after planting) which result in the emergence of primaries are not clear. Dowson (1926) was unable to reproduce primary symptoms by artificially inoculating bulbs with *B. narcissicola* mycelium. However, Price (1978) infected foliage at emergence by placing sclerotia in the old flower stalk base within the bulb neck. Gray (1971) suggested that after planting infected bulbs there was rapid growth of the pathogen to the bulb neck and infection of the emerging scale leaves (the leaf sheath). Later, Gray and Shiel (1975) noted lines of mycelium resembling *B. narcissicola* in scale leaves; they suggested that the fungus transfers from scale leaves to foliage leaves, but only invades those leaves which are damaged; or healthy leaves during cold, wet weather. However, various combinations of damage, chilling, high moisture levels and low oxygen levels applied to the bulb neck did not alter the subsequent incidences of infection in either bulbs or leaves.

Infected bulbs may not be the sole inoculum source responsible for shoots emerging with smoulder. As with tulip fire, it is quite plausible that shoot infections could arise from infected leaf fragments or bulb tissue remaining on or in the soil from a previous season (Westerdijk, 1916; Gregory, 1937; Moore, 1939). For stocks left down for two or more seasons the procedures of flailing after foliage dieback and re-ridging will presumably introduce and spread debris within the soil. In field trials on cultivation

techniques that might control smoulder, Hardwick, Chadburn and Millar (1978) found that disease incidence increased significantly when infected debris was added to ridges.

Conidia produced on shoots emerging with smoulder are an immediate inoculum for secondary spread of the disease. Moore (1979) stated that germinating sclerotia provide an additional inoculum in the form of ascospores. Although Gregory (1941) induced apothecia in south-west England, similar experiments by Gray and Shiel (1975) in Scotland were not successful. Spores are probably dispersed by wind and water-splash onto neighbouring plants (Moore 1979) and presumably may lead to secondary infection. Gray (1971) observed bulb mites (Rhizoglyphus echinopus) detaching conidia from sclerotia and bulb scales and suggested that they play a part in dispersal of the fungus.

In contrast to tulip fire no distinct phase of leaf spotting has been reported after the emergence of smoulder primaries. Only Klebahn (1907) has described leaf spotting and this was probably a primary symptom rather than secondary infection. Anon. (1978) reported that leaf and flower spotting were very rare. One factor which may have a bearing on the lack of a distinct leaf-spotting phase is the reported inability of B. narcissicola to invade healthy narcissus tissue (Klebahn, 1907; Dowson, 1924; Gray, 1971); conidia and sclerotia were able to infect only wounded or senescent tissue. Beaumont (1935) suggested that damage during cultivation (eg hoeing) assisted spread of the disease and more recently Hardwick et al. (1977a) noted sporulation of botrytis on open stalk ends left after flower

picking.

Considering the apparent inability of B. narcissicola to infect healthy narcissus tissue, the means by which secondary spread occurs remains somewhat speculative. Assuming that secondary infection does occur, the infection cycle would be completed by deposition of B. narcissicola in the soil, as sclerotia or on infected litter, and/or by the infection of healthy bulbs.

Moore (1979) stated that sclerotia develop in infected leaves showing dieback and they remain on or in the soil. The length of time which infected litter or sclerotia of B. narcissicola remain a danger to emerging narcissus shoots in subsequent seasons are not known. The sclerotia of B. tulipae remain viable for less than two seasons after burial (Coley-Smith and Javed, 1972) while those of Rhizoctonia tuliparum can remain viable for at least 10 years (Coley-Smith, Humphrey-Jones and Gladders, 1979). Botrytis conidia probably survive burial in soil for a period shorter than that of sclerotia (Park, 1955). Under humid conditions mycelium of B. cinerea may survive storage for more than one year (Van den Berg and Lentz, 1968).

Healthy bulbs could be infected through several routes, for example, from mother to daughter bulb. Price (1978) has shown that in severe cases of bulb neck rot a lesion containing B. narcissicola could extend to infect an adjacent, daughter bulb within the cluster. A second possible pathway is the movement or growth of B. narcissicola through the soil from adjacent, infected bulb clusters or from sclerotia in the soil. Klebahn (1907) observed the

development of botrytis sporulation on bulbs close to where sclerotia had been placed in the soil. A third possible pathway is the dispersal of conidia from infected leaves or stems into the bulb neck and Gray et al. (1975) suggested that conidia were indeed carried into the bulb neck by water or mites to infect bulb tissue damaged by bulb scale mites. Other alternatives are that the fungus grows down into the bulb from leaves or stems infected after damage. Beaumont (1935) reported that B. narcissicola often grew down into the bulb where it caused a yellow-brown decay of the base and scales. Moore (1979) also suggested that sclerotia developing at the bulb neck results from downward growth of mycelium. By contrast Gregory (1937), stated that although B. narcissicola could grow from infected leaves to the bulb, it did not usually do so and Gray and Shiel (1975) observed that mycelium did not spread beyond leaf abscission zones into bulb tissue. A final pathway leading to bulb infection is by contamination, with debris or conidia from infected plants at lifting and grading or during storage. Whichever pathway(s) operate in the field, inoculations are most likely to be successful if a bulb is predisposed to infection by injury or stress (Hawker, 1940; Gray and Shiel, 1975).

Various aspects of bulb cultivation may influence the incidence of smoulder; these include the location of planting site, the time of planting and the length of time for which a stock is left in the ground. McWhorter and Weiss (1932) and Gray (1971) found higher frequencies of smoulder on heavy soils than on soils with good natural drainage. The former suggested that growers should avoid

low, wet sites, heavy soils and areas subject to frost and dew. Gray and Shiel (1975) found an increased incidence of smoulder where forcing boxes had stood on an impervious base rather than on ash and when infected bulbs were transplanted into puddled soil. Beaumont (1935) stated that B. narcissicola was most active when temperatures were low and growth of narcissus slow. Moore (1939) also noted that smoulder was most prevalent during cold, wet seasons and occurred frequently in early flowering districts.

It has been noted in several studies (McWhorter and Weiss, 1932; Gray and Shiel, 1975; Humphreys-Jones, 1975) that smoulder is rarely seen in first year plantings but increases considerably in the second and subsequent years.

2. Control

Moore (1939) stated that there was no known cure for smoulder. He advised that attempts to control the level of infection in bulb stocks should concentrate on prevention - by careful inspection of bulbs before planting and by roguing after emergence. He suggested that all diseased plants should be removed and burnt. Gray (1971), however, reported that commercial growers in north-east Scotland had not controlled smoulder by roguing and that repeated, thorough roguing of experimental plots did not affect the level of bulb infection.

As there is some evidence to suggest that smoulder is bulb-borne, attempts have been made to control the disease by various bulb treatments. Some growers believe that smoulder is reduced if the outer, membranous scales, on which

sclerotia occur, are removed before replanting (Moore, 1979). Several fungicide treatments have been tested. Bulbs are usually given a hot-water treatment (HWT) after lifting to control stem eelworm; the addition of formaldehyde and/or organo-mercuric fungicides to the hot-water bath, or given as a cold steep after HWT, does not appear to control smoulder (McWhorter and Weiss, 1932, Hawker, 1940; Gray, 1971). Hawker suggested that such treatments failed because the sclerotia survived. Gray (1971) also noted that commercial growers obtained no control by the application of quintozene to bulbs or to the soil and Hardwick et al. (1977b) were similarly unsuccessful with double soil drenches of benomyl, thiram, quintozene, dicloran, dichlofluanid, iprodione or urea. Gray and Shiel (1975), however, observed fewer shoots with smoulder if bulbs bearing sclerotia were dipped in 0.1% benomyl before planting.

Variable results have also been reported after using fungicide sprays in attempts to control smoulder. Gray (1971) reported that growers in northern Scotland had obtained no control using thiram or zineb sprays and in field trials she failed to control smoulder with sprays of dichlofluanid or mancozeb/zineb. In East Anglia, however, Briggs (1972) reported that a regular spray with mancozeb/zineb, from full foliage emergence to mid-June, considerably reduced aerial spread of B. narcissicola into plots of healthy Verger plants, although it had no effect on an infected stock.

Varietal resistance to smoulder has not been reported. Dowson (1926) tested five cultivars by inoculation and all were susceptible to B. narcissicola. Gregory (1937) noted

that reports of considerable bulb rotting in store frequently involved Narcissus poeticus varieties.

SECTION III

MECHANISMS OF RESISTANCE

Although numerous studies of the mechanisms by which plants resist infection by fungi have been made, most have been limited to a few major crop plants. Investigations into the resistance mechanisms of bulbous plants have been confined to the tulip (Bergman, 1966; Bergman, Beijersbergen, Overeem and Sijpesteijn, 1967; Schonbeck and Schroder, 1972) and the onion (Walker and Stahmann, 1955; Clark and Lorbeer, 1973) and narcissus has not previously been examined.

Resistance to fungal infection in plants is often classified by either structural or chemical features and these in turn are subdivided into either pre-infectional (passive or constitutive) or post-infectional (active or induced) phenomena (Ingham, 1973). Although these categories are not exclusive, for example lignification may be considered as the production of a structural and/or a chemical barrier, they form a useful framework for describing mechanisms of resistance.

1. Pre-infectional, structural resistance

The subject has been reviewed by Brown (1936) and Hart (1949) and more recently by Wood (1967) and Royle (1976). Many reports have associated pre-infectional, structural features with resistance to infection but few have provided convincing evidence for causal relationships. Features often quoted as examples of pre-infectional resistance are the thickness or hardness of the cuticle, the size and shape of stomata,

and leaf waxiness and hairiness. A 'mechanical theory' of rust resistance was proposed as long ago as 1892 by Cobb. Recently, Jennings (1962) has shown correlations between morphological features of raspberry canes that hinder inoculum deposition (hairiness, spininess and waxiness) and resistance to grey mould (B. cinerea), spur blight (Didymella aplanata) and canespot (Elsinoe veneta).

Martin (1964) reviewed the role of the cuticle in plant diseases and concluded that it was not an important barrier to penetration, either physical or chemical.

Stomata, by their abundance, location, morphology and function, may influence the entry of pathogens into a plant. Differences in number may confine infection to a particular leaf surface; for example, the adaxial leaf surface for hop and grapevine downy mildews. Royle (1976) found correlations between the number of open stomata on hops and the level of downy mildew (Pseudoperonospora humuli) infection seven days later. Leaf surface topography and a chemical factor associated with phytosynthesis were shown to encourage zoospores to select open rather than closed stomata on which to settle, encyst, and germinate quickly. On wheat Puccinia graminis is simply less able to penetrate closed rather than open stomata (Burrage, 1970).

Once a pathogen has gained entry into a plant, structural features may limit its development. Restriction of sporulation in Colletotrichum infections has been attributed to wall strength (Marks, Berbee and Riker, 1965). The importance of lignified and suberized cell walls in containing invasion has long been recognised (Hursch, 1924);

such barriers are thought to become more important as plants age (Paxton and Chamberlain, 1969).

2. Post-infectional structural resistance (cell wall alterations)

Post-infectional structural resistance includes lignification, the development of cork barriers, tyloses, wound gums and lignitubers (Ingham, 1973). Here, I shall describe examples of alterations to the cell wall. Evidence for cell wall alterations occurring in response to infection has recently increased (Ride, 1978), with methods other than histological staining being employed.

Since the work of Young (1926) it has been known that wall alterations may take many forms - swelling, deposition of material on the outside or inside of cells (reaction material, papillae, callosities or sheaths) or the production of a series of new walls by rapid cell division. Frequently, the materials involved in wall modifications are reported to be lignin, occasionally callose and, less commonly, suberin, melanin, calcium or silicon.

Evidence that cell wall lignification is associated with resistance has been presented for several host-parasite interactions. Lignification in cucumbers infected by Cladosporium cucumerinum was first reported by Behr (1949) and subsequently investigated more fully by Hijwegen (1963). Asada and Matsumoto have used both biochemical and histological techniques to demonstrate lignification in Japanese radish infected with Peronospora parasitica (Asada and Matsumoto, 1969, 1971, 1972). Ride (1975) and Ride and Pearce (1979) provided convincing evidence for lignification

in wheat leaves inoculated with B. cinerea and other non-pathogens. Van Maarschalkerweerd and Verhoeff (1976) reported thickened cell walls and an increase in a lignin-like material following infection of tomato fruits by B. cinerea (ghost spot). A resistance response which involves lignification has also been described in leaves and tubers of potato (Friend, 1976) and leaves of reed canarygrass and other species of the Gramineae (Sherwood and Vance, 1976, 1980).

The production of papillae and related wound plugs was reviewed by Aist (1976). Callose is a common and often major component of such structures. Other components include cellulose, protein, pectin, suberin, gums and silicon. Sherwood and Vance (1976) showed the incorporation of lignin into pre-existing papillae.

In some plants ions accumulate in cell walls following infection; thus, calcium was reported in bean hypocotyls infected with Rhizoctonia solani (Bateman, 1964) and in apple fruits infected with Venturia inaequalis (Shear and Drake, 1971). Kunoh and Ishizaki (1975, 1976) reported accumulation of silicon near fungal penetration sites in leaves of wheat, barley, cucumber and morning glory.

The resistance of plants to vascular wilt pathogens may involve both cell wall modification and chemical inhibitors (e.g. Mace, 1978); the former often includes suberisation and the development of lignitubers and tyloses. Wardlaw (1930) suggested that in the Panama wilt disease of banana, caused by Fusarium oxysporum f. sp. cubense, mechanical processes at the root base (suberised cambiform

layers, tyloses and collapsed xylem vessels) could account for resistance in 90% of infected roots.

3. Pre-infectional chemical resistance (prohibition)

Four types of chemical resistance were recognised by Ingham (1973) - prohibitins, inhibitins, post-inhibitins and phytoalexins. Although the terms proposed for the different categories imply roles in resistance which in many cases have not been proved, they help to rationalise the numerous examples of antifungal chemicals found in plants. By definition, only compounds of the first class (prohibitins) are present in tissues before infection at levels sufficient to provide a degree of resistance. The other three classes require a marked increase in amount following infection in order to establish a fungitoxic environment and are therefore described in the following section on post-infectional (active) chemical resistance. The occurrence of pre-existing antimicrobial substances in plants and their role in disease resistance was reveiwed by Overeem (1976).

Ingham (1973) defined a prohibitin as a "pre-infectional plant metabolite which can markedly reduce or completely halt the *in vivo* development of an organism unadapted to its effects". A well-documented example is the resistance of onion bulbs to *Colletotrichum circinans*. The dead, outer bulb scales of resistant cultivars contain two antifungal phenolics: protocatechuic acid (3,4-dihydroxybenzoic acid) and catechol (3,4-dihydroxybenzene). These are water-soluble and diffuse into infection droplets preventing spore germination. If the outer scales are removed from a bulb, the inner, fleshy scales are readily invaded by the pathogen (Walker and Stahmann, 1955).

More recently, antifungal substances have been isolated from leaf surfaces. The compounds are mainly di- or triterpenoids and methylated flavonoids. Bailey, Vincent and Burden (1974) found two highly fungitoxic diterpenoids, sclareol and episclareol, associated with tobacco leaves. Harborne, Ingham, King and Payne (1976) described two isoflavones, luteone and wighteone, on and in Lupinus albus leaves. In both examples the concentration of toxic compounds found on leaves appeared sufficient to provide a barrier to fungal invasion.

Other compounds which may be considered prohibitins are the alkaloids solanine and tomatine, found in potato and tomato leaves respectively (Schlösser, 1975), and the saponin cyclamin in cyclamen leaves (Schlösser, 1971). Gripenberg (1948) ascribed the decay resistance of Western red cedar heartwood to the presence of three fungitoxic compounds, the thujaplicins. Fungitoxic compounds are probably present in other woods resistant to fungal decay (Overeem, 1976).

4. Post-infectional chemical resistance

A. Inhibitins

These were defined by Ingham as "pre-infectional plant metabolites which, although present in detectable quantities in apparently healthy plants, must undergo a marked post-infectional increase if their toxic potential is to be fully expressed". The localised accumulation of antifungal compounds at sites of attempted infection would seem to be a more effective means of defence than a passive chemical barrier (e.g. a toxin on the leaf surface or a

prohibitin), which is of little use once bypassed.

Aromatic compounds with antifungal activity frequently accumulate at sites of attempted infection. Minamikawa, Akazawa and Uritani (1963) described the accumulation of a fungitoxic coumarin derivative, scopoletin, following infection of sweet potatoes by Ceratocystis fimbriata. In Irish potatoes, Hughes and Swain (1960) noted an increase in scopolin (scopoletin-7-glucoside) and chlorogenic acid following infection by Phytophthora infestans. Sakuma, Yoshihara and Sakamura (1976) described the accumulation of antifungal phenolic compounds in red clover tissue following infection by Kabatiella caulivora.

B. Post-inhibitins

Ingham (1973) defined post-inhibitins as "antimicrobial metabolites produced by plants in response to infection (or mechanical or chemical damage) but whose formation does not involve the elaboration of a biosynthetic pathway within the tissue of the host". Post-inhibitins are present at toxic levels before infection but are in an inactive or bound form. The active molecules are frequently released by enzymic hydrolysis from an inactive glycoside precursor. Numerous examples are known.

The resistance of young tulip bulbs to Fusarium oxysporum f.sp. tulipae has been attributed to post-inhibitins. Bergman (1966) isolated a fungitoxic compound, tulipalin A, from the white skin of young bulbs. The compound, identified as α-methylene-γ-butyrolactone, was subsequently found as the less active glycoside, tuliposide A (Bergman et al., 1967; Bergman and Beijersbergen, 1968). The level of tuliposide A

in bulbs decreased towards the end of the growing season as the outer, white skin turned brown and papery; at the same time infection by F. oxysporum f.sp. tulipae increased (Bergman and Beijersbergen, 1968). A related glucoside which also readily converts to a fungitoxic lactone, tuliposide B, was isolated from the pistil, stalk and leaves of tulip plants (Schönbeck and Schroeder, 1972).

Schönbeck and Schroeder (1972) examined the role of tuliposides in the specificity of infection of tulips by species of Botrytis. Botrytis tulipae is a major pathogen of tulips while the ubiquitous B. cinerea causes little damage. Although B. cinerea can infect, it soon stops growing. Schönbeck and Schroeder (1972) found that B. cinerea was considerably more sensitive to tuliposides than B. tulipae; the former converted the glycoside to the toxic lactone while the latter converted it to the non-toxic acid. It was also found that B. cinerea increased the permeability of cell membranes of tulips to a greater extent than B. tulipae, so presumably the former fungus caused the more rapid release of tuliposides.

Tulipalin A and several closely related lactones have been isolated from other members of the Liliaceae (Cavallito and Haskell, 1946; Slob, Jekel, Jong and Schlatmann, 1975) and some members of the Ranunculaceae (Hill and van Heyningen, 1951; Benn and Yelland, 1968). Recently, fungitoxic lactones were found in the stems of wild avocado trees (Persea borbonia) and possibly have a role in their resistance to Phytophthora cinnamomi (Zaki, Zentmyer, Pettus, Sims, Keen and Sing, 1980).

In addition to the lactones, several other types of fungitoxic compounds which are released from precursors and accumulate on infection appear to have a role in disease resistance. These include saponins in oat roots (Turner, 1961; Burkhardt, Maizel and Mitchell, 1964) and in ivy leaves (Schlösser, 1973), allyl isothiocyanates in Brassica oleracea (Greenhalgh and Mitchell, 1976), hydrogen cyanide in birdsfoot trefoil (Millar and Higgins, 1970; Fry and Millar 1971 a,b), a phenolic quinone in apple leaves (Overeem, 1976) and benzoxazolines in the roots of rye, wheat and maize (Overeem, 1976; Deverall, 1976).

C. Phytoalexins

Induced chemical resistance in plants was first proposed at the turn of the century. Restriction of mycorrhizal fungi in orchids was one subject of early investigations which led to such proposals (Bernard, 1909, 1911). In 1933, Chester critically reviewed work on acquired physiological immunity in plants and made pertinent suggestions as to the directions future research should take. The term phytoalexins was introduced by Müller and Borger (1941) following work on the resistance of potato tubers to Phytophthora infestans. Müller (1956) defined phytoalexins as "antibiotics which are the result of an interaction of two different metabolic systems, the host and the parasite, and which inhibit the growth of microorganisms pathogenic to plants". Ingham (1973) modified this definition to include non-microbial induction. He also distinguished phytoalexins from post-inhibitins according to whether the antimicrobial compound accumulated as a result of de novo synthesis or was released from a precursor. The term phytoalexin was thus restricted

to, "an antibiotic formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors". As yet, however, knowledge of the biosynthesis of most phytoalexins is poor; some synthetic pathways have been demonstrated but control enzymes have not been identified. No studies have shown de novo synthesis. Thus, the evidence for according an antimicrobial compound the term phytoalexin is the relative slowness of accumulation (hours rather than minutes) and the lack of knowledge of simple precursors (Deverall, 1976).

In the last two decades, phytoalexins have been extensively investigated. Their isolation and characterisation, biosynthesis, localisation, mode of action, metabolism and more recently induction (or elicitation) have all been studied as aspects of a more general problem - their role in disease resistance. Reviews on phytoalexins are numerous (Cruickshank, 1963; Cruickshank, Biggs and Perrin, 1971; Ingham, 1972; Kuć, 1972; Deverall, 1972, 1976, 1977; Kuć, 1976; Kuć, Currier and Shih, 1976; Van Etten and Pueppke, 1976; Keen and Bruegger, 1977; Harborne and Ingham, 1978).

Phytoalexins are generally compounds of low molecular weight (250-500), frequently phenolics, and include a wide range of chemical families. The 91 compounds listed by Harborne and Ingham (1978) covered 20 families and ranged from benzoic acid to complex stilbene oligomers.

Since 1978, newly characterised phytoalexins have included further furanoacetylenic compounds from Vicia faba (Mansfield, Porter and Smallman, 1980), an acetylenic compound

(falcarindol) from tomato leaves (Garrod, Lewis and Coxon, 1978), an arylbenzofuran from Coronilla emerus (Dewick and Ingham, 1980), isoflavans from Lotus hispidus and Astragalus cicer (Ingham and Dewick, 1979, 1980) and a stilbene from vine leaves (Langcake, Cornford and Pryce, 1979). Thus, there are now c. 100 fully characterised phytoalexins. Numerous other reports have described phytoalexin accumulation within infected tissue but the compounds involved await purification and identification.

Phytoalexins have been described from some 75 species, representing 20 families (Keen and Bruegger, 1977). In most cases the compounds produced by a given plant species are the same irrespective of the nature of the elicitor. With the exception of some species of pine (Shain, 1967; Hills and Inoue, 1968) and possibly Ginkgo biloba (Christensen and Sproston, 1972) phytoalexin production appears confined to the angiosperms. Within the angiosperms many families have still not been investigated; most of the phytoalexins so far characterised have been isolated from species of only two families, the Leguminoseae and the Solanaceae. The Cucurbitaceae appear exceptional in that no phytoalexins have been found despite thorough investigation (Harborne and Ingham, 1978).

Phytoalexins produced by species of one family are often of similar chemical structure. Members of the Leguminoseae are characterised by isoflavonoid compounds (Van Etten and Pueppke, 1976) and the Solanaceae by sesquiterpenoid compounds (Kuć et al., 1976; Gross, 1979). Although fewer species have been investigated, the Malvaceae appear to be characterised by napthaldehydes and naphthafurans, the

Convolvulaceae by furanoterpenoids, the Orchidaceae by phenanthrenes and the Umbelliferae possibly by furanocoumarins (Harborne and Ingham, 1978).

From monocotyledenous Angiosperms only five phytoalexins have been characterised; two diterpenoid compounds (momilactones) from rice (Cartwright, Langcake, Pryce and Leworthy, 1977) and three phenanthrene derivatives, orchinol, hircinol and loroglossol, from orchid bulbs (Hardegger, Billand and Corrodi, 1963; Gaumann, 1964; Fisch, Flick and Arditti, 1973; Ward, Unwin and Stoessl, 1975).

MATERIALS AND METHODS

SECTION I

GENERAL

1. Plant material

A. Source and storage of bulbs

Bulbs of Golden Harvest, the cultivar used in all experiments unless stated otherwise, were purchased from Grays Garden Shop, Stirling, each September. Bulbs required for pathogenicity tests and studies on resistance were stored at 4°C.

B. Growth of plants

Within 5 days of purchase bulbs were hand-planted c. 15cm deep and c. 10cm apart at the University gardens, Stirling. No chemical treatments were applied to the growing plants. Leaves were removed for pathogenicity tests as required.

2. Fungi

A. Source of Botrytis isolates and other fungi

Ten isolates of *B. cinerea*, originally obtained from plants other than narcissus, and single isolates of *B. allii*, *B. elliptica*, *B. fabae* and *B. tulipae* were supplied by Dr. J.W. Mansfield (University of Stirling). *Botrytis narcissicola* isolates 11A, 12B, D1, D2, D3 and D4 were supplied by Dr. G.D. Lyon, Scottish Horticultural Research Institute (SHRI), Dundee and isolates PP525, PP526 and PP527 by Dr. N.V. Hardwick, Agricultural Development and Advisory Service, Derby. Dr. J.S.W. Dickens, Plant Pathology Laboratory, Harpenden, provided isolates of

B. narcissicola (R15C) and *B. cinerea* (A64), both obtained from narcissus, whose identities were confirmed by the Commonwealth Mycological Institute (CMI), Kew. Additional isolates of *B. narcissicola* and *B. cinerea* were collected from field grown narcissus at several sites (Section II.1). Cultures of *Cladosporium herbarum* were obtained from the Stirling University Culture Collection (SCC).

B. Culture and storage of fungi

Isolates of *Botrytis* and *C. herbarum* were induced to sporulate by growing them on Medium X (Last and Hamley, 1956) under longwave UV radiation (Phillips 'Black Light' fluorescent tubes, 16h photoperiod) at 18°C for 6-10 days. Conical flasks (250ml) containing *c.* 40ml of the medium were inoculated with a small piece of agar from a sporulating culture after wetting its surface with sterile distilled water (SDW). For sclerotia production, isolates of *Botrytis* were grown on potato dextrose agar (PDA) plates at 18°C in the dark for 4-6 weeks. To maintain pathogenicity of *Botrytis* cultures, selected isolates were stored as suspensions of mycelial fragments and conidia in 10% (v/v) glycerol/water under liquid nitrogen. Volumes of *c.* 1ml were sealed in glass ampoules and stored in a 'Vivostat' (British Oxygen Company). Ampoules were thawed and suspensions pipetted into flasks of Medium X when required for pathogenicity tests or studies on infection and resistance.

C. Preparation of conidial suspensions

Spores were harvested by flooding sporulating cultures with SDW and lightly scraping the surface with a sterile needle. The resultant suspension was filtered through muslin and

the spores washed, to remove nutrients carried over from the agar, by three cycles of centrifuging (1000g for 2 mins) in SDW. Following counts with a haemocytometer, final suspensions were adjusted to c. 10^5 spores per ml of SDW or other appropriate liquid as required.

3. Culture media

A. Medium X

Solution (i)		Solution (ii)	
Glucose	10g	$MgSO_4.7H_2O$	0.5g
Mycological peptone	2g	Distilled water	100ml
Casein hydroysate	3g		
KH_2PO_4	1.5g	Solution (iii)	
$NaNO_3$	6g	Oxoid agar No. 3	30g
KCl	0.5g	Distilled water	700ml
Yeast nucleic acid	0.5g		
Distilled water	200ml		

Solutions (i) and (ii) were prepared while (iii) was steamed. When the agar had dissolved (i) (ii) and (iii) were mixed. Aliquots (40ml) were dispensed into 250ml conical flasks and autoclaved for 15 min at 1 Kg/cm^2.

B. V8 juice agar (acidified)

Contents:		
	V8 juice (Campbells Soups Ltd.)	200ml
	Distilled water	800ml
	Oxoid agar No. 3	30g

The agar and distilled water were placed in a steamer until the agar had dissolved. V8 juice was added and the pH adjusted to 6.0 with sodium hydroxide (NaOH). Aliquots were dispensed into glass bottles and autoclaved as above.

Plates were poured when the bottles had cooled. Where necessary, streptomycin sulphate (Sigma) was added to bottles of cooled agar to give a concentration of 100 µg/ml.

C. Potato dextrose agar (PDA)

Contents:		
	Oxoid potato dextrose agar	39g
	Distilled water	1000ml

The agar and distilled water were placed in a steamer until the agar had dissolved. Aliquots were dispensed into glass bottles and autoclaved for 15 min at 1 Kg/cm^2. Plates were poured when the bottles had cooled.

D. Czapek Dox liquid medium

Contents:		
	Oxoid Czapek Dox liquid medium	33.4g
	Distilled water	1000ml

The medium and distilled water were thoroughly mixed, dispensed into glass bottles and autoclaved as above.

E. Synthetic Pod Nutrients (SPN)

Contents:		
	Sucrose	5g
	Casamino acids	380mg
	KH_2PO_4	100mg
	$MgSO_4.7H_2O$	50mg
	Distilled water	1000ml

The pH of the medium was adjusted to 4.0 with galacturonic acid before being dispensed into glass bottles and autoclaved as above.

SECTION II

THE CAUSE OF SMOULDER AND THE INFECTION OF NARCISSUS BY SPECIES OF *BOTRYTIS*

1. *Isolation of* Botrytis *from field-grown plants*

Botrytis narcissicola and *B. cinerea* were isolated from field-grown plants showing a variety of symptoms. For each symptom samples were taken from at least 10 and usually *c*. 30 plants. Small pieces of tissue, *c*. 2x2mm, dissected from the edge of a lesion, containing flecks or other symptoms, were surface sterilized in sodium hypochlorite (NaOCl) and plated onto V8 juice agar containing streptomycin sulphate (150 units/ml). Tissue samples were surface sterilized by immersion in NaOCl (1% available chlorine) for 3 mins followed by two rinses in SDW. Where present, *Botrytis* grew out of tissue samples after incubation for 5 days at 18°C. With sporulating lesions, either as found in the field or induced by incubation on moistened tissue paper in a sandwich box, spores were transferred directly to the said medium. Isolates were sub-cultured on PDA to assist with species identification (Section 1.1). Apparently healthy leaves and senescent leaves were also sampled for the presence of *Botrytis* by isolation. Sclerotia removed from bulbs or lesions were surface sterilized (as above) before plating onto V8 juice agar.

2. *Pathogenicity tests*

A. *Preparation of leaf and bulb tissue*

Mature leaves from field-grown narcissus were lightly washed, dried gently with tissue paper and placed adaxial surface uppermost on moist tissue paper in plastic sandwich

boxes. A wick of tissue paper was wrapped around the cut leaf end. Fleshy bulb tissues of the second generation (see Appendix 1) were dissected from bulbs and placed with the adaxial surface uppermost in moistened sandwich boxes.

B. Inoculation

Conidial inocula consisted of 20 µl droplets of suspension of 10^5 spores/ml in SDW. Mycelial inocula were 5mm diameter discs taken from the edge of young cultures on V8 juice agar; these were inverted onto the tissue surface. Mycelial cultures were grown in the dark to suppress sporulation. A minimum of 10 leaves or bulb scales and 10 inocula were used in each test and all experiments, unless stated otherwise, were repeated.

C. Incubation

Closed boxes of inoculated tissue were incubated in a growth cabinet (Gallenkamp) at 18 $\pm$ 1^{o}C and illuminated for 16h each day by fluorescent tubes (Phillips Coolwhite).

D. Assessment of infection

After incubation for 5 days inoculations were recorded as having caused either no symptoms, a limited lesion or a spreading lesion. These categories are described in more detail in Chapter 1.

SECTION III

SMOULDER EPIDEMIOLOGY

1. Infected bulbs

A. Sites and cultivation

(i) Scottish Horticultural Research Institute. In August 1977 c. 500 bulbs, cv. Verger, from a stock of narcissus with a history of smoulder, were planted mechanically c. 20cm deep, at 15cm intervals in ridges 71cm apart in a well-drained loam soil. After foliage dieback in autumn 1978 the plot was re-ridged and bulbs were left in the ground for a second season. Weeds were controlled by linuron (1.12 Kg/ha), paraquat (3 l/ha) and chlorophan 2.2 l/ha) applied in early December.

(ii) Commercial fields, Laurencekirk. Plantings of narcissus at Cushnie Farm, Laurencekirk (Mr. R.S.M. Milne) were examined in spring 1979. A field of cv. Golden Harvest planted two seasons previously (autumn 1976) was chosen for detailed observations on smoulder symptoms and for experiments on epidemiology. Fungicides were not applied.

(iii) Virus-tested (VT) stocks at Aberdeen, Dundee and Edinburgh. Stocks of VT narcissus being multiplied by twin-scaling propagation at the North of Scotland College of Agriculture (NOSCA), Aberdeen, at SHRI, Dundee and at the East of Scotland College of Agriculture (ESCA), Edinburgh were examined in 1979 for symptoms of smoulder. The cultivation of narcissus during twin-scale propagation has been described, among others, by Mowat and Chambers (1975),

Hanks and Rees (1978, 1979) and Turner (1979). Cultivation at NOSCA, ESCA and SHRI is summarised below.

First year bulbs were grown in a peat compost in frost-protected glasshouses; second and third year bulbs were grown in gauze (Tygan) houses. At NOSCA and SHRI the growing medium was Universal compost and at ESCA soil was sterilised with methyl bromide and given a top dressing of pure peat compost. Flowers were removed after inspection and at NOSCA trash was removed by hand after the foliage had died back. Weeds were controlled by hand-weeding (NOSCA) or hand-weeding supplemented by pre- and post-emergence herbicides. Mother bulbs for twin-scaling were dipped for 30 mins in a suspension of benomyl (0.2% w/v) but no fungicides were applied thereafter.

B. Estimation of the incidence of symptoms

In the plot of bulbs (cv. Verger) at SHRI, individual plants were identified by position, counting from the ends of each 6m ridge. As an additional check, some plants were marked with a numbered stake. All plants were examined for smoulder at regular intervals during 1978 and 1979.

In the commercial plantings, all shoots in five randomly selected 10m lengths of ridge were examined. Shoots with more than one symptom, a rare occurrence, were placed in the category of the most extensive symptom on that shoot. The total number of shoots in each 10m length of ridge was estimated from counts of two 1m lengths within each ridge.

2. Artificial inoculation of field-grown plants

A. Source of bulbs

Fourteen hundred bulbs (1000 of cv. Sempre Avanti, 400 of cv. Golden Harvest), 12-15cm diameter, were purchased from Grampian Growers Ltd., Montrose. Each bulb was examined and those with botrytis sclerotia in the outer, papery scales were excluded from the main planting stock. Two hundred single-nosed bulbs, cv. Sempre Avanti, obtained by twin-scaling propagation from a VT mother bulb, were donated by Mr. W. P. Mowat, SHRI. One hundred of these were 3-4cm diameter and the remainder 4-6cm.

B. Cultivation

Plants were grown in a well-drained loam soil at SHRI in a plot where narcissus had not been cultivated previously; the preceeding crop was potatoes. For each infection experiment, bulbs of one cultivar were positioned at 15cm intervals in furrows 71cm apart and the furrows ridged mechanically. The smaller (VT) bulbs were hand-planted 10cm deep and 15cm apart in ridges. Bulbs were stored at 12^{o}C until planting (23/10/78). Plants for shoot inoculation experiments were examined at emergence and the few with smoulder symptoms were discarded. Weeds were controlled by handweeding and by herbicide sprays (as in Section 1A(i); no fungicides were applied. The plot was re-ridged in August 1979, leaving bulbs in the ground for a second season.

C. Experimental design

A plot of 22 ridges by 20m was divided into eight sub-plots, each corresponding to one infection experiment (Fig. A).

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Fourteen hundred bulbs (1000 of cv. Sempre Avanti, 400 of cv. Golden Harvest), 12-15cm diameter, were purchased from Grampian Growers Ltd., Montrose. Each bulb was examined and those with botrytis sclerotia in the outer, papery scales were excluded from the main planting stock. Two hundred single-nosed bulbs, cv. Sempre Avanti, obtained by twin-scaling propagation from a VT mother bulb, were donated by Mr. W. P. Mowat, SHRI. One hundred of these were 3-4cm diameter and the remainder 4-6cm.

B. Cultivation

Plants were grown in a well-drained loam soil at SHRI in a plot where narcissus had not been cultivated previously; the preceeding crop was potatoes. For each infection experiment, bulbs of one cultivar were positioned at 15cm intervals in furrows 71cm apart and the furrows ridged mechanically. The smaller (VT) bulbs were hand-planted 10cm deep and 15cm apart in ridges. Bulbs were stored at 12^{o}C until planting (23/10/78). Plants for shoot inoculation experiments were examined at emergence and the few with smoulder symptoms were discarded. Weeds were controlled by handweeding and by herbicide sprays (as in Section 1A(i); no fungicides were applied. The plot was re-ridged in August 1979, leaving bulbs in the ground for a second season.

C. Experimental design

A plot of 22 ridges by 20m was divided into eight sub-plots, each corresponding to one infection experiment (Fig. A).

Non-inoculated plants were grown between adjacent plots and in perimeter guard rows.

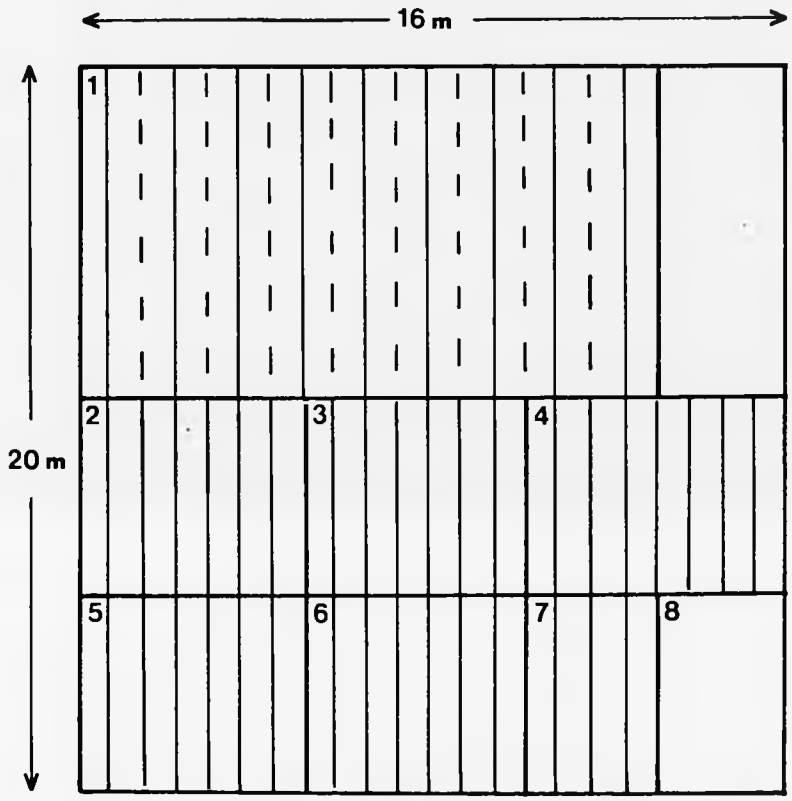


FIG. A. Layout of plots (1-8) for experiments investigating infection by B. narcissicola. Plants grown at the perimeter and between adjacent plots were not inoculated (——). In plot 1, where blocks of plants were spray-inoculated with conidia, empty ridges (— —) were left between ridges of inoculated plants (———). In plot 2, stalk ends were inoculated with conidia and in plot 3 wound-inoculated bulbs were grown. Plants in plot 4 were inoculated on the leaves with mycelium. In plot 5, scale debris bearing sclerotia was placed in the soil above virus-tested bulbs. Bulbs bearing sclerotia were planted in plot 6. Virus-tested bulbs were inoculated with mycelium in plot 7. The survival of sclerotia buried in the soil was investigated in plot 8. In plots 2-6, treatments were assigned to plants at random, with each treatment applied to 48 plants; 24 plants were used for each treatment in plot 7. In plot 1, each treatment (8 in all) was applied to a group of six plants in each ridge.

D. Inoculation of shoots

Individual plants were spray-inoculated with 2ml of a suspension of conidia (10^5/ml) in SDW or V8 juice (25% v/v; pH 6) using a Shandon spray unit. Where necessary, plants were wounded by making three horizontal scratches with a needle on the adaxial surface of five leaves. Leaves were inoculated with mycelium by inverting agar discs (8mm) bearing mycelium onto the adaxial leaf surfaces, inocula being held in position with Sellotape. One disc, sited towards the middle of a mature leaf and c. 5cm above soil level, was applied to each plant. For inoculation of flower stalks, one flower bud was cut from each plant leaving a stump of c. 2cm. Exuded sap was removed with a tissue and the open stalk end inoculated with 0.5ml of a suspension of $5x10^5$ spores/ml from a Pasteur pipette. Appropriate control inocula were included in each experiment. All inoculated tissues were enclosed in a polythene bag for 24h following inoculation.

E. Inoculation of VT bulbs

Bulbs were inoculated by positioning discs (5mm) of agar bearing mycelium on fleshy scale tissue at the bulb neck. The inocula were held in position by the surrounding soil when the bulbs were hand-planted.

F. Production of infected bulbs

To obtain planting stock uniformly infected with B. narcissicola, bulbs were wound-inoculated. A plug of bulb tissue 2-4mm thick was removed with a 5mm diameter cork borer and mycelial inocula on agar inserted into the wounds, mycelium innermost. After incubation for 4 days at $18^{\circ}C$

in moist polythene bags most inocula had caused brown lesions c. 2cm across, in the outer scales. The inocula were allowed to dry out before planting by storing bulbs for 3 days at 18°C.

G. Assessment of infection

Inoculated shoots were examined for symptoms immediately after removal of polythene bags, 1 wk after inoculation, and subsequently at c. 2 wk intervals. Inoculated bulbs were scored for emergence and the shoots examined for symptoms (April-May, 1979). All bulbs were left in the ground for a second year and the shoots examined for infection after emergence (March, 1980). Plants were later dug up (April, 1980) and examined for sheath lesions. Differences in numbers of infected plants were tested for significance using χ^2.

3. The survival of B. narcissicola mycelium in bulbs

Lesions were established in small (3-4cm) VT bulbs, cv. Sempre Avanti, by inoculating wounds with B. narcissicola mycelium. The lesions were restricted when c. 1cm in diameter by drying for 3 days at 18°C. Bulbs were then stored in the laboratory over moist or dry tissue paper in plastic sandwich boxes enclosed in loosely-tied polythene bags. Isolation confirmed that viable B. narcissicola remained in the restricted lesions at the start of the storage period. Small portions of tissue (c. 2x2mm) at the edge of lesions were sampled for B. narcissicola after 6 wks storage.

4. The survival of B. narcissicola sclerotia buried in soil

A. Production, burial and recovery of sclerotia

Sclerotia of B. narcissicola (isolate 11A) were produced by growing cultures on PDA at 18°C in the dark. After 6 wks sclerotia were removed with forceps, washed three times in SDW to remove nutrients carried over from the agar, and air-dried. Batches of 20 sclerotia were mixed with 10g of washed silver sand and placed in small bags made from 68µm mesh nylon fabric (Henry-Simon Ltd., Stockport). Nylon bags are resistant to microbial attack and the mesh size chosen allowed passage of fungal hyphae while retaining the sclerotia. Thirty-six bags of sclerotia were buried in a loam soil at SHRI in December 1978; the bags were positioned 20cm apart in a 1x1m square. A similar experiment was initiated in September 1979 using sclerotia grown on autoclaved narcissus leaves and less sand (2g) per bag. At intervals of c. 6 wks (experiment 1) or 12 wks (experiment 2) three replicate bags were removed. The chosen particle size of sand (< 400µm) was smaller than that of B. narcissicola sclerotia and the latter were readily recovered by sieving. The number of sclerotia recovered from each bag was counted and all were examined under a dissecting microscope for evidence of germination.

B. Viability test

From each bag one half of the sclerotia were rinsed thrice in SDW and the remainder were surface sterilised in NaOCl (as for tissue isolations, Section II.1). Individual sclerotia were aseptically plated onto small discs cut from a layer of V8 juice agar amended with streptomycin sulphate

(100µg/ml). Petri dishes containing 10 sclerotia, each on individual agar discs, were incubated at 18°C in the dark and after 5 days the number of sclerotia producing a mycelium was recorded. Preliminary experiments indicated that 18°C was the optimum temperature for rapid germination of viable sclerotia which usually occurred within 3 days. The method of placing sclerotia on individual discs of agar (Illman, 1960; Coley-Smith and Javed, 1970) avoids the problem of slowly germinating sclerotia being overgrown by mycelium from those germinating rapidly.

5. Influence of the depth and time of bulb planting on the incidence of smoulder primaries

A. Source of bulbs

Plants with a history of smoulder during 1978 or 1979, selected from the plot of cv. Verger at SHRI, were hand-harvested in August 1979, cleaned and dried. The bulbs were sorted into three size grades; 8-10cm (120), 10-12cm (80) and 12-14cm (120) and each grade lot was divided into four portions. Four treatments were investigated, with a total of 80 bulbs (30+20+30) in each treatment. The majority of bulbs were single-nosed; double-nosed bulbs, mainly 12-14cm, were distributed equally among the treatments.

B. Cultivation

Bulbs were hand-planted in a well-drained loam soil at SHRI on 13/9/79 at depths of 5, 15 and 25cm (from bulb nose to soil surface) and on 15/12/79 at 15cm. The bulbs were spaced 30cm apart each way in a bed c. 6x5m surrounded by perimeter guard rows.

C. Experimental design

Each of four treatments was applied to 80 replicate blocks of four bulbs. Treatments within blocks were assigned at random.

6. Influence of the growing medium on the incidence of primaries

Bulbs of plants with a history of smoulder, selected from the plot of cv. Verger at SHRI, were planted in peat, coarse sand and steam-sterilised loam. For each treatment 40 bulbs (16-18cm) were planted, one per 20cm pot, with a depth of 10cm between bulb noses and the soil surface. Single and double nosed bulbs were distributed equally among the treatments. The pots were sunk to the rim in soil (18/9/79) at Stirling University gardens in 40 replicate blocks of three pots, treatments within blocks being randomised. The plants were examined in spring for primary symptoms of smoulder.

SECTION IV

MECHANISMS OF RESISTANCE TO *BOTRYTIS* IN NARCISSUS BULBS

1. Inoculation

A. Bulb scales

Experiments on the resistance of bulbs to *Botrytis* were performed with cv. Golden Harvest unless otherwise stated.

Bulb scales were prepared, inoculated and incubated as described for pathogenicity tests (Section I.2) except that more inocula were placed on each scale. In some experiments the epidermis was removed with a pair of watchmaker's forceps prior to inoculation to facilitate excision of infected tissue.

B. Glass slides

Before use, glass slides were placed in stainless steel racks and thoroughly cleaned. After soaking in a surface active detergent (Haemo-sol) for 24h they were rinsed under running tap water and finally with distilled water (3x) and dried at 120°C. This cleaning technique gave slides which had no deleterious effects on spore germination or spread of bioassay droplets. Clean slides were supported on test tubes in closed plastic sandwich boxes (17x11x5cm) lined with moist tissue paper. Three 20 µl droplets of a spore suspension were pipetted onto each slide and the boxes then incubated at 18°C in the dark.

2. Infection development

A. Preparation of epidermal strips for examination

Shallow cuts in the form of a square were made with a razor blade around inoculation sites and the epidermal strips underlying inocula were removed. Detached strips were mounted on glass slides in water for observation of spore germination, germ tube growth and cellular response by the plant.

B. Microscopy

Examinations of inoculum droplets on glass slides and preliminary observations of epidermal strips were made with a Wild microscope. Further examinations of epidermal strips were made by transmission fluorescence microscopy (Reichart Fluoropan Microscope; excitation filter BG12, emission filters OG1 and GG9) and by bright field and interference contrast microscopy (Zeiss Research Microscope with Nomarski optics). Micrographs of fluorescing material were obtained using 0.5-2.0 min. exposures of Pan F film (Ilford).

C. Quantitative assessment of infection development

Growth of fungi in epidermal strips and on glass slides was stopped at intervals by the addition to inoculum droplets of a drop of cotton blue in lactophenol stain (0.0067% w/v; Anon., 1968). Germination, considered to be the production of a germ tube of any length, was assessed by counts of 100 spores. At each assessment, four replicate droplets were examined and the germ tube length of 24 germinated spores was measured using a calibrated micrometer eyepiece.

3. Histochemical tests

A. For lignin

(i) Toluidine blue (0.05% w/v) in 0.1M phosphate buffer, pH 6.8. Epidermal strips were immersed in the stain for c. 2 mins. Lignified walls stain green or bluish-green (O'Brien, Feder and McCully, 1964).

(ii) Azure B (0.025%) in citrate buffer, pH 4.0. Epidermal strips were immersed in the stain for c. 5 mins., then rinsed in distilled water. Cell walls containing lignin stain green (Jensen, 1962).

(iii) Phloroglucinol-hydrochloric acid (Wiesner test). Two methods were used. In the first, tissue was soaked in a saturated (1% w/v) aqueous solution of phloroglucinol in 20% HCl. Lignified tissue rapidly develops a red-violet colour (Siegel, 1953; Jensen, 1962). In the second method, tissue was soaked in a 2% (w/v) solution of phloroglucinol in 95% EtOH for 1-2h., and then dipped in conc. HCl for 30s. Lignified tissue stains a red-purple colour (Sass, 1968).

(iv) Chlorine-sulphite. Tissue was chlorinated by immersion in a fresh, saturated, acidified solution of calcium hypochlorite for 5 mins., and then treated with a cold solution of sodium sulphite (1% w/v). Lignified tissue develops a bright red colour in a few minutes which gradually (30-40 mins.) fades to a brown hue (Campbell, Bryant and Swann, 1937; Jensen, 1962).

(v) Autofluorescence. Epidermal strips were mounted in water and viewed with UV light (366nm). Lignin and wall-bound phenolics fluoresce various colours ranging from

yellow to blue-green (Harris and Hartley, 1976).

B. For callose

(i) Aniline blue (0.005% w/v) in 50% EtOH. Tissue was immersed in the stain for 4-24h and then rinsed in water; callose stains blue (Currier, 1957; Jensen, 1962).

(ii) Aniline blue fluorescence. Tissue was immersed in water} aniline soluble blue (0.005% w/v) in 0.15M phosphate buffer, pH 8.2 for 10 mins. Callose fluoresces yellow with UV light (Currier and Strugger, 1956; Jensen, 1962).

(iii) Lacmoid (0.1% w/v) in 50% EtOH. Tissue was immersed in stain for 30 mins and then rinsed in water; callose stains blue (Jensen, 1962; Reynold and Dashek, 1976).

C. Assessment of fungal death

(i) Trypan blue (1% w/v). A small drop (c. 10µl) of stain was added to detached epidermal strips mounted in water or to sporeling bioassay droplets and slides were examined after 5 mins. Dead cytoplasm stains dark blue (Rossall, Mansfield and Hutson, 1980).

D. Assessment of host cell death

(i) Fluorescein diacetate. Fluorescein diacetate was dissolved in acetone (5mg/ml) and diluted with water to 0.01% w/v. Tissue was immersed in the stain for 5 mins. before observation with UV light. Live cells accumulate fluorescein which fluoresces green-yellow (Rotman and Papermaster, 1966; Widholm, 1972).

(ii) Evans blue (0.5% w/v). Tissue was immersed in the stain for 5 mins. Dead cells stain dark blue (Turner and Novacky, 1974).

(iii) Plasmolysis. Tissue was mounted in a 1M sucrose solution of neutral red (0.1% w/v). Live cells plasmolyse and neutral red aids their recognition.

E. Microautoradiography

The procedure described by Ride and Pearce (1979) was closely followed. Bulb scales were inoculated with either a spore suspension of B. cinerea or SDW and incubated in the dark at 20°C. After 24h, inoculation droplets were removed and a radioactive solution was injected into bulb tissue, beneath the epidermis close to inoculation sites, from a 1ml syringe. The injection solution consisted of [3-^{14}C] cinnamic acid (Fluorochem Ltd., Glossop, Derby; 58 mCi/mmol) prepared as a 0.5 mCi/ml solution in 0.05M phosphate buffer at pH 6.0. After infiltration of label, inoculum droplets were replaced and tissues incubated for a further 24h. Epidermal strips were peeled off and extracted with hot (80°C) 70% EtOH for 15 mins followed by hot water (80°C) for 15 mins. Strips were mounted on glass slides, inoculated surface uppermost, and allowed to dry overnight. Small pieces of film (Ilford Pan F) were placed over the dry epidermal strips and held in place with a second glass slide. Piles of slides were secured with elastic bands, placed in black polythene bags and stored at 4°C. After 3 days exposure the images were developed.

4. Chemicals

A. Solvents for tissue extraction, gel filtration and thin layer chromatography (TLC)

All solvents were Analar grade except for hexane which was laboratory reagent grade. Diethyl ether was distilled

before use to remove antifungal compounds previously noted in some batches (Hargreaves, 1976).

B. Solvents for high pressure liquid chromatography (HPLC)

All solvents were HPLC grade. Water was distilled in glass from potassium permanganate to remove phthalates (Porter, Smallman and Mansfield, 1979). Solvents were de-gassed before use by boiling under vacuum for a few minutes.

C. TLC spray reagents

(i) Diazotised p-nitroaniline (DpNA) for phenolics.

Spray solution: 2ml p-nitroaniline (0.5 w/v) in 2M HCl, 5 drops of sodium nitrite (0.5% w/v) and 8ml of sodium acetate (20% w/v).

After spraying the chromatograms with a freshly prepared solution phenolic compounds form products of various colours (Swain, 1953; Ribereau-Gayon, 1972).

(ii) Aluminium chloride ($AlCl_3$) for flavonoids

Spray solution: 1% (w/v) ethanolic solution of $AlCl_3$.

Flavonoids fluoresce yellow under long wave UV radiation (Merck, 1971).

(iii) Vanillin - sulphuric acid (H_2SO_4) for higher alcohols, phenols, steroids and essential oils.

Spray solution: 3% (w/v) ethanolic solution of vanillin containing 0.5ml conc. H_2SO_4.

Higher alcohols give a blue colour on chromatograms sprayed and heated to 120°C (Merck, 1971)

(iv) Hydroxylamine hydrochloride - iron(III)chloride ($NH_2OH.HCl$-$FeCl_3$) for lactones, esters, amides and anhydrides of carboxylic acids.

Spray solutions: (a) 20g $NH_2OH.HCl$ dissolved in 50ml water,

and made up to 200ml with EtOH; (b) 50g KOH dissolved in a little water and made up to 500ml with EtOH. Solutions (a) and (b) were mixed in equal parts and the precipitated KCl filtered off (solution 1). 10g powdered $FeCl_3$ was dissolved in 20ml conc. HCl and shaken with 200ml of Et_2O until a homogeneous mixture was obtained (solution 2). Plates were sprayed with solution 1, dried at room temperature, and sprayed with solution 2. Carboxylic acid derivatives show up as purplish spots (Whittaker and Wijesundera, 1952; Merck, 1971).

(v) 2,4-Dinitrophenylhydrazine (2,4-DNP) for carbonyl groups. Spray solutions: (a) 0.4% (v/v) 2,4-DNP in 2M HCl; (b) 0.2% (w/v) potassium hexacyanoferrate(III) in 2M HCl. Chromatograms were sprayed with solution (a) and then (b). Saturated ketone derivatives turn blue immediately, saturated aldehyde derivatives turn green more slowly and unsaturated carbonyl derivatives rarely develop a colour (Merck, 1971).

D. Source of flavonoid compounds

Flavone, flavanone, chrysin, naringenin and naringin were purchased from Aldrich Chemical Co., Gillingham, Dorset and epicatechin from Sigma Chemical Co., Glasgow.
Dr. D.T. Coxon, ARC Food Research Institute, Norwich, synthesised 7-hydroxyflavan, 7,4'-dihydroxyflavan, 7,4'-dihydroxy-8-methylflavan and the corresponding flavylium chloride salts (Coxon, O'Neill, Mansfield and Porter, 1980). Dr. A.E.A. Porter, University of Stirling, synthesised 4-hydroxyflavan. Other flavonoid compounds were kindly supplied by Dr. R.G. Cooke, University of Melbourne,

Australia (7-methoxy-4'-hydroxy-8-methylflavan), Professor D.G. Roux, University of The Orange Free State, South Africa (7,4'-dimethoxyflavan, isoflavans NA37-47, fisetinidol and mollisacacidin), Dr. J.L. Ingham, University of Reading (liquiritigenin) and Dr. G.M. Barton, Canadian Forestry Service, Vancouver, Canada (poriol).

5. Measurement of pH

Values of pH were measured with a Pye Unicam model PW9418 pH meter.

6. Spectral analyses

Ultraviolet (UV) absorption spectra were obtained with a Pye Unicam SP1800 spectrophotometer.

Mass spectra (MS) and nuclear magnetic resonance spectra (NMR) were obtained and analysed by either Dr. D.T. Coxon, Food Research Institute, Norwich, or Dr. D. Dance, Chemistry Department, University of Stirling.

7. Preparation of bulb scale extracts

A. Collection of diffusates and tissue

Inoculum droplets (diffusates) were collected with a Pasteur pipette. Tissue was scraped from inoculum sites with a scalpel blade. Diffusates and tissue were collected over ice and, if not extracted immediately, stored at -20°C.

B. Extraction procedures

Precautions were taken to minimise exposing extracts to light.

Excised tissue was homogenised in a Sorval omnimixer (three 15s bursts at half maximum speed) in redistilled Et_2O (at least 10ml Et_2O/g fr.wt. tissue) and left to soak at 4°C for 3h. The ethereal supernatant was decanted and the homogenate washed twice with Et_2O. Following Et_2O extraction some tissues were subsequently re-extracted with MeOH and/or amyl alcohol (AmOH). Bulked extracts and washings were dried over anhydrous sodium sulphate, centrifuged (5 min at 850g), evaporated in vacuo at 30°C (Buchi Rotavapor R) and then stored under oxygen-free nitrogen at -20°C.

Diffusates were partitioned three times with equal volumes of Et_2O in 50ml test tubes. The ethereal and aqueous phases were mixed by agitation with a 'whirly-mixer'. Bulked Et_2O extracts were dried as above.

8. Bioassay techniques

A. TLC plate bioassays

The method devised by Klarman and Sanford (1968) was used to detect antifungal compounds in thin layer chromatograms of extracts. Volumes equivalent to the extract from 0.2 or 0.4g fr. wt. of inoculated tissue were applied. Developed chromatograms were sprayed with a dense suspension of C. herbarum spores in Czapek Dox liquid medium and incubated at 25°C in moist chambers for 4 days. The fungus had been grown on Medium X for 8-10d at 18°C. Inhibitory compounds were revealed as areas of white silica gel where the dark green fungus failed to grow.

Solutions of purified compounds in MeOH or chloroform ($CHCl_3$) were spotted onto TLC places (20µl on small areas,

c. 30mm^2) using drawn-out Pasteur pipettes. Their antifungal activity was assessed on a semi-quantitative basis according to the clarity and extent of inhibition zones (on a scale of 0 to 4; see footnote to Table 3.22).

B. Sporeling bioassays

The microscope-slide bioassay for antifungal activity devised by Purkayastha and Deverall (1965b) and modified by Hargreaves, Mansfield and Rossall (1977) was used. Extracts and compounds were assayed against germ tube growth of pre-germinated B. cinerea and B. narcissicola spores. Bulb tissue extracts were usually tested at a concentration equivalent to the extract from 0.2g fr.wt. of inoculated tissue per ml.

Glass slides were cleaned as previously described. The required amount of extract or compound to be assayed was dissolved in dimethylsulphoxide (DMSO) and added to sterile SPN (pH 4) or Czapek Dox (pH 6.8) liquid medium; final DMSO concentrations were adjusted to 2% (v/v). Three 20µl droplets (replicates) of suspensions of botrytis conidia in sterile nutrient solution (2.5 x 10^4 spores/ml) were pipetted onto individual slides and conidia were allowed to germinate for 6h. After 6h most conidia had developed germ tubes 30-50µm in length and the sporelings adhered to the glass slides. Droplets were removed by absorption with filter paper and replaced by nutrient solution (20µl) containing the test compound or extract. As a control, some droplets were replaced with a nutrient solution containing 2% DMSO alone. After incubation at 18°C for a further 18h sporelings were killed and stained by adding a drop of

cotton blue in lactophenol. The germ tube lengths of 20 sporelings per droplet were measured with a calibrated micrometer eyepiece (if < 100µm) or with a map recorder from camera lucida drawings; the total length of all germ tubes produced by each conidium was recorded. Results were expressed as replicate means. Variation between replicates was invariably low. Inhibition of sporeling growth (%) was calculated by comparison with growth in the nutrient solution lacking a test compound.

Growth of some sporelings in Czapek Dox stopped when solutions were changed after 6h; the sporelings of limited growth (30-50µm) usually occurred at the circumference of droplets. A modified assay was therefore devised to preclude the possibility of sporelings dying by drying out. Test solutions were adjusted to twice the required final concentration in 4% DMSO and 20µl droplets were then added to drops of similar volume containing pre-germinated spores. Sporelings were killed and germ tube lengths measured after incubation at 18°C for a further 12h. This modified assay is described in the text as the 'solution addition' assay.

C. Paper disc bioassay for antibacterial activity

The method used was similar to that described by Gnanamanickam and Smith (1980). Antibiotic assay discs (Whatman, 6mm diam.) were loaded with 50µg of the test compound in 25µl MeOH. Discs were dried for at least 1h before transfer to Petri dishes containing nutrient agar (Oxoid) overlaid with soft agar (nutrient broth with 0.75% agar) and seeded with the test bacterium. Each bacterial strain had been grown at 30°C

for 16h in nutrient broth. One hundred µl of the selected culture containing c. 10^8 cells/ml was added to 2ml of molten soft agar (45°C), mixed thoroughly and poured over the already hardened nutrient agar surface as a uniform layer. Inhibition zones were measured after incubation for 24h at 28°C.

9. Search for preformed chemical inhibitors

A. Invasion of frozen-thawed and leached tissue by B. narcissicola and B. cinerea

Bulb scales were frozen (-20°C) and slowly thawed (4°C) to disrupt membrane integrity. Half of the frozen-thawed scales were leached (3x) by soaking them for 1h in 500ml of distilled water. Healthy, frozen-thawed and frozen-thawed-leached bulb scales were inoculated with mycelium of B. narcissicola and B. cinerea and incubated at 18°C. Invasion of tissue was assessed visually according to the extent and density of aerial mycelium produced.

B. Bioassay of extracts from healthy (non-inoculated) tissue

Non-inoculated bulb scales and separated epidermal and mesophyll bulb scale tissues were extracted with Et_2O and MeOH. Tissues were extracted directly and after a freeze-thaw treatment (-20°C for 12h then 18°C for 6h) to test both for preformed inhibitors and for antifungal compounds released on tissue damage. Extracts were assayed on TLC plates with C. herbarum.

In a further experiment, bulb scale epidermal and mesophyll tissues were hydrolysed in hot acid (40 min in 2M HCl at 100°C). Aglycones were removed from the cooled

acid hydrolysate by partition (3x) with ethyl acetate and AmOH (Ribereau-Gayon, 1972). Extracts were tested for antifungal activity as above.

10. Detection of phytoalexin accumulation

A. In diffusates

Bulb scales were inoculated with SDW or a B. cinerea conidial suspension and diffusates collected after 24h. The bulked diffusates were divided into two portions in the ratio of 2:1 (v/v), and the larger volumes filtered (Millipore filter, 45µm). Half the volume of each filtrate was extracted with Et_2O. The antifungal activity of crude, filtered and filtered-extracted diffusates was assessed against B. cinerea conidia in SDW.

B. In tissue

Bulb scales with the epidermis removed were inoculated with SDW or a B. cinerea conidial suspension and tissues extracted after incubation for three days. Thin layer chromatograms of Et_2O and MeOH extracts were assayed with C. herbarum.

11. Fractionation of extracts and isolation of phytoalexins

A. TLC

(i) Analytical. Extracts were applied (1.5cm origin) to pre-coated TLC plates (Merck 5715, Si gel F_{254}, 0.25mm thick) using drawn-out Pasteur pipettes. For two-directional separation, extracts were spotted onto a plate 2cm from one corner. Chromatograms were developed in a range of solvents by ascending chromatography. Plates were dried and examined under 254 and 366nm UV light (Universal

lamp, Camag) and bands observed marked with a pencil.

(ii) Preparative. Preparative thin layer chromatography (PC) was carried out on pre-coated plates (as above). Extracts from c. 1g fr.wt. of tissue were applied per cm of origin and chromatograms were developed in either Et_2O-petrol (2:1) or hexane-acetone (2:1). Bands were scraped from plates with a scalpel blade and compounds eluted with MeOH and Et_2O. Silica gel was removed from eluates by centrifugation (5 mins at 850g) and the eluates were evaporated to dryness.

All chromatograms were developed in closed chromatography tanks lined with tissue paper soaked in solvent.

B. Gel filtration

For isolation of milligram quantities of phytoalexins, the extract from tissues collected 5 days after inoculation of stripped scales with B. cinerea conidia was initially fractionated by gel filtration through a 70 x 2.5cm column of LH20 Sephadex (Pharmacia Ltd.), eluting with MeOH. Sephadex powder was left to swell overnight in MeOH and the column slurry-packed. Before the start of an experiment the column was flushed with MeOH for c. 2h. The flow rate was adjusted to 1 or 2 ml/min by restriction of the outlet tube. The Et_2O extract from c. 50g fr.wt. of tissue was applied in MeOH (5ml) and elution was monitored spectrophotometrically at 254nm. Fractions were collected at 10 min intervals and a sample of each, equivalent to 0.1g fr.wt. of tissue, was spotted onto a TLC plate and assayed with C. herbarum. Samples of selected antifungal fractions,

equivalent to 1.0g fr.wt. of tissue, were further examined by TLC in Et_2O-petrol (2:1) and chromatograms assayed with C. herbarum.

C. HPLC

Hydroxyflavan phytoalexins were purified and crude extracts analysed by reversed-phase HPLC. The liquid chromatography system consisted of two Waters Associates pumps (Models 6000 and 6000A) controlled by a solvent programmer (Model 660). A 20 x 0.8cm stainless steel column slurry-packed with 5µm ODS Hypersil (Shandon, London) and fitted with an on-column needle-through-septum injector (Bristow, 1976) was connected to the detector flow cell (10µl) by 60mm of microbore (15µm) capillary tubing. Detection was by UV absorption with a Cecil Model 272 variable wavelength spectrophotometer. The eluent reservoirs were contained in a water bath held at 30°C and water from this bath was circulated through a jacket surrounding the column.

For purification of hydroxyflavan phytoalexins, volumes of partially purified compounds (total of c. 2mg) were injected in 30µl MeOH and eluted isocratically at 5 ml/min with 35% MeOH in 5% HCO_2H.

For a one-step fractionation of crude extracts, a sample equivalent to 1g fr.wt. of infected tissue was injected in 25µl MeOH and eluted as above. The eluate was collected in fractions corresponding to major UV absorption peaks, groups of small peaks, or hollows, and each fraction was evaporated to dryness and resuspended in 0.25ml MeOH. Volumes of 25µl (0.1g fr.wt. of tissue) were spotted onto

a TLC plate and assayed with C. herbarum; selected antifungal fractions were assayed against B. cinerea sporelings in SPN at 0.1, 0.5 and 1.0g fr.wt./ml.

Samples of crude extracts (c. 0.1-1.0g fr. wt. in 1-10µl MeOH) were subsequently analysed by isocratic and gradient elutions with a range of solvent mixtures. A semi-quantitative estimate of the amounts of hydroxyflavan phytoalexins in different extracts was made from measurements of peak area, using as an approximation the equation: peak area = peak height x peak width at base x 0.5.

EXPERIMENTAL WORK AND RESULTS

CHAPTER 1

THE CAUSE OF SMOULDER AND THE INFECTION OF NARCISSUS BY SPECIES OF *BOTRYTIS*

The aim of the work described in this chapter was to identify species of *Botrytis* found associated with narcissus and to compare the pathogenecities of isolates of *B. narcissicola*, *B. cinerea* and other spp. of *Botrytis* towards narcissus.

1. *Identification of* B. narcissicola *and* B. cinerea

The presence of botrytis sporulation or sclerotia on narcissus does not necessarily indicate infection by the host-specific pathogen *B. narcissicola*. *B. cinerea* can readily invade wounded or senescent tissue of many plant species and is a potential cause of narcissus smoulder. It is important therefore to distinguish between these two species if their respective roles in the etiology of smoulder are to be understood. A third *Botrytis* sp., *B. polyblastis*, the cause of narcissus fire, is readily distinguished from the other two species by its large conidia (Dowson, 1928b). The morphology of conidia, conidiophores and sclerotia are commonly used in the identification of *Botrytis* spp. (Ellis, 1971), as is the pathogenicity of an isolate towards tissue of a suspected host.

A. *Morphology*

Isolates of *Botrytis* collected from field-grown narcissus with disease symptoms or from senescent tissue, were

cultured on PDA and Medium X and sclerotia, conidia and conidiophores were examined to distinguish B. narcissicola and B. cinerea.

(i) Sclerotia. Sclerotia produced on PDA were of two types; small (0.5-1.5mm diameter) almost spherical sclerotia, evenly distributed across plates, or much larger (3-4mm diameter) with irregular shape and frequently produced in an annular pattern. By comparison with isolates identified by CMI as B. narcissicola or B. cinerea it was found that the small sclerotial type corresponded to B. narcissicola and the large to B. cinerea. Although sclerotia of the latter varied considerably in size, shape and pattern of formation they were readily distinguished from those of B. narcissicola which were consistently small and developed all over plates of PDA (Plate 1.1). For certain isolates the lengths and widths of 20 sclerotia were measured using an eye-piece graticule in a binocular microscope; the results (Table 1.1) illustrate the clear differences between the two species.

(ii) Conidia. Conidia harvested from 10 day old cultures on Medium X were mounted on glass slides in water and immediately observed at X100 magnification. For each of 10 isolates of (a) B. narcissicola isolated from narcissus, (b) B. cinerea isolated from narcissus and (c) B. cinerea isolated from other plant species, the lengths and widths of 40 conidia were measured. The results are presented in Figure 1.1 with isolates arranged in order of increasing conidial length for each set. Conidia from B. narcissicola were generally longer and thinner than those from B. cinerea; median values for the three sets of isolates were

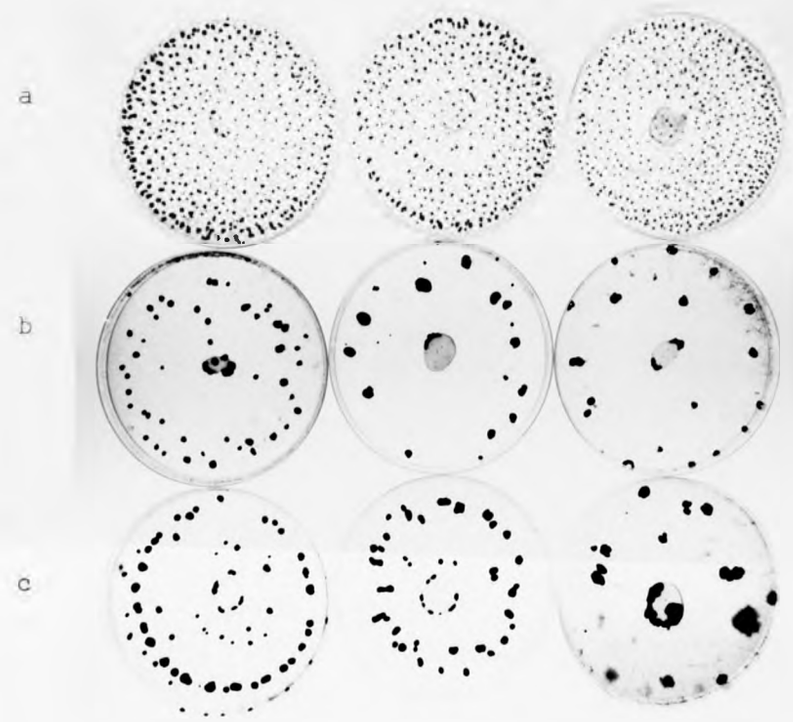


PLATE 1.1 Sclerotia produced on PDA by isolates of B. narcissicola (a) and B. cinerea (b and c). Isolates of B. narcissicola (79N6, 526, 527) were obtained from narcissus and isolates of B. cinerea from narcissus (78C4, 78C6, 79C6; row b) and other hosts (pea, vine and raspberry; row c).

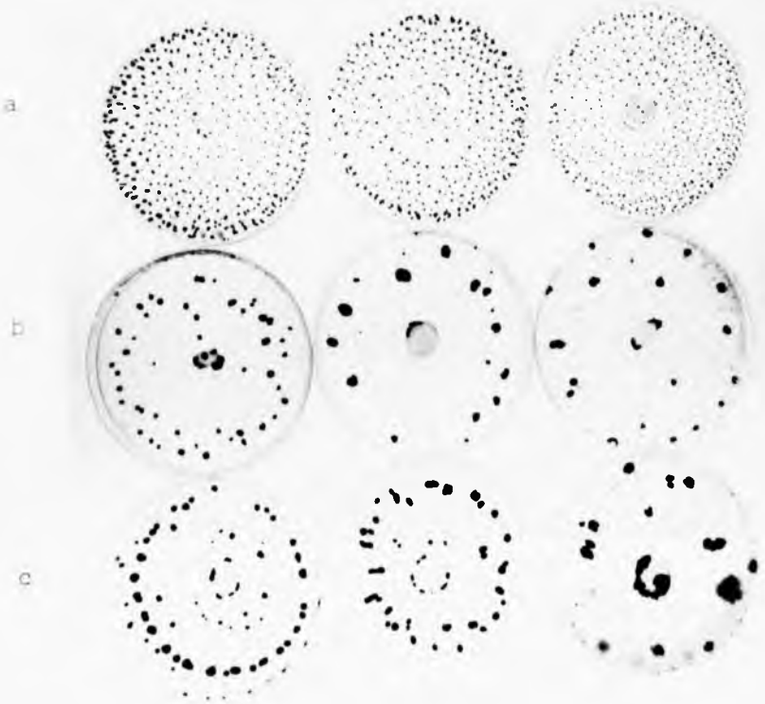


PLATE 1.1 Sclerotia produced on PDA by isolates of B. narcissicola (a) and B. cinerea (b and c). Isolates of B. narcissicola (79N6, 526, 527) were obtained from narcissus and isolates of B. cinerea from narcissus (78C4, 78C6, 79C6; row b) and other hosts (pea, vine and raspberry; row c).

TABLE 1.1 Size of sclerotia produced by isolates of B. cinerea and B. narcissicola on PDA

Species	Isolate	Source	Size (mm)[a] Length	Width
B. cinerea	BC39	Field bean	3.2±0.7	2.3±0.4
	J27	Field bean	4.4±0.5	3.0±0.4
	JM5	Potato	3.9±0.4	2.9±0.3
	J14	Raspberry	4.1±0.4	3.3±0.2
	B232	Tomato	3.5±0.4	2.7±0.4
B. cinerea	A64	Narcissus	2.5±0.2	2.1±0.2
	78C2	Narcissus	3.2 ±0.5	2.3±0.3
	78C4	Narcissus	3.6±0.5	2.7±0.4
	78C6	Narcissus	2.3±0.3	2.0±0.2
	79C3	Narcissus	3.2±0.5	2.4±0.3
B. narcissicola	11A	Narcissus	1.6±0.1	1.3±0.2
	D4	Narcissus		
	525	Narcissus	1.3±0.1	1.0±0.1
	526	Narcissus	1.4±0.1	1.0±0.1
	R15C	Narcissus	1.7±0.2	1.3±0.1

[a]Mean ± 95% confidence limits.

FIG. 1.1 Size of *B. cinerea* and *B. narcissicola* conidia. The lengths and widths of (a), ten isolates of *B. cinerea* collected from narcissus (b), ten isolates of *B. cinerea* collected from other plants and (c), ten isolates of *B. narcissicola* collected from narcissus are shown. Dimensions are expressed as the mean (of 40) with 95% confidence limits. Within each set of ten, the isolates are arranged in order of increasing conidial length. Note that although the means of *B. narcissicola* conidial lengths are generally greater than those of *B. cinerea*, there is considerable overlap between the two species.

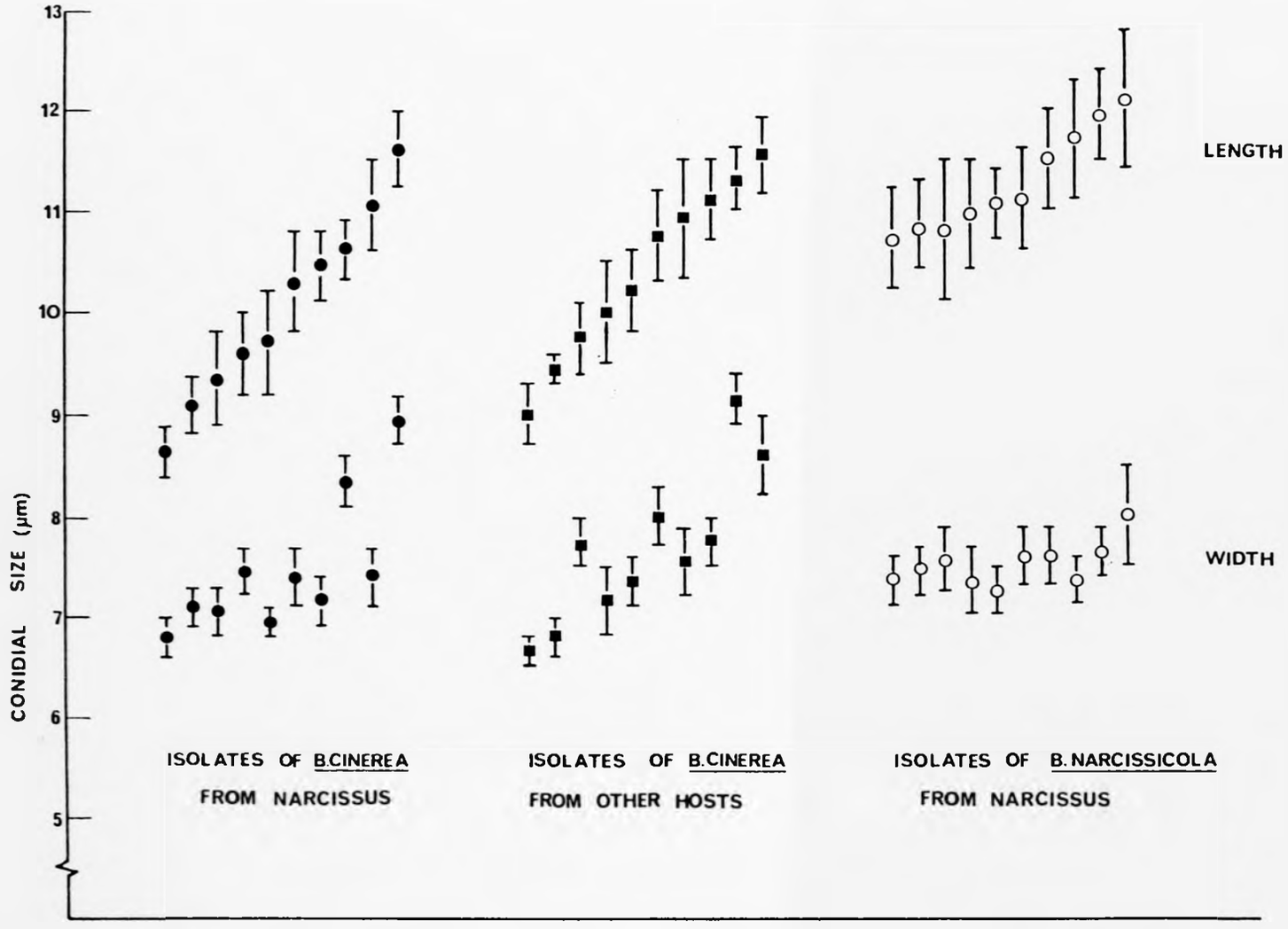

13
12
11
10
9
8
7
6
5
CONIDIAL SIZE (μm)
LENGTH
WIDTH
ISOLATES OF B.CINEREA FROM NARCISSUS
ISOLATES OF B.CINEREA FROM OTHER HOSTS
ISOLATES OF B.NARCISSICOLA FROM NARCISSUS

FIG. 1.1

(length x width) (a) 11.0 x 7.4 µm, (b) 9.6 x 7.5 µm and (c) 10.0 x 7.2 µm. The variation in size between isolates, particularly of B. cinerea, was so considerable that the range of conidial lengths for the two species overlapped, precluding the use of conidial size as a sole means of identification.

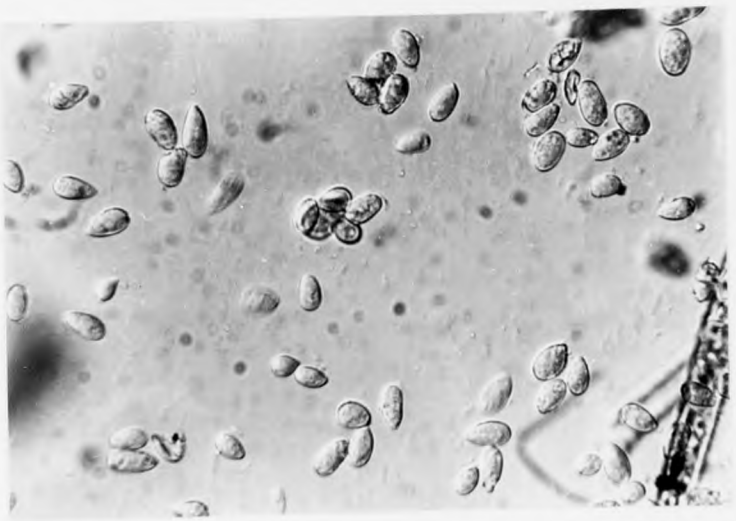
B. narcissicola conidia tended to be pear-shaped while those of B. cinerea were oval (Plate 1.2) but again variation, particularly within B. cinerea isolates, precluded the use of shape as an aid to identification.

(iii) Conidiophores. To investigate conidiophore structure, 10 dayold sporulating cultures were examined with a binocular microscope and pieces of sporulating mycelium were mounted in water on slides. All B. cinerea isolates and the majority of B. narcissicola isolates were found to have branched conidiophores. The two species differed slightly in that branching of B. narcissicola conidiophores, unlike B. cinerea, was largely restricted to the conidiophore tip (Table 1.2). Plate 1.3 illustrates the similarity of morphology which precluded the use of conidiophore branching as a reliable distinguishing character.

B. Pathogenicity

Isolates of Botrytis collected from narcissus and identified as either B. narcissicola or B. cinerea on the basis of sclerotial morphology were tested for their pathogenicity towards detached bulb and leaf tissue using conidial and mycelial inocula (Table 1.3). The bulb surface below conidial inocula of both B. narcissicola and B. cinerea

a.



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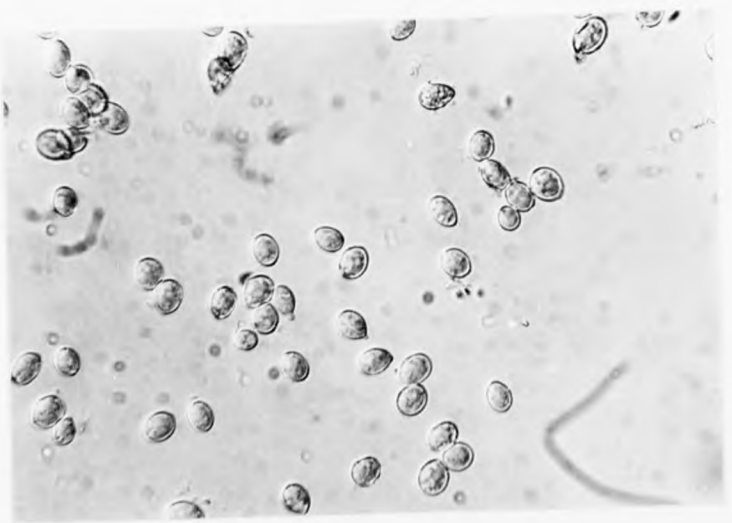


PLATE 1.2 Conidia of *B. narcissicola* (a) and *B. cinerea* (b) (x 600).

a.

b.

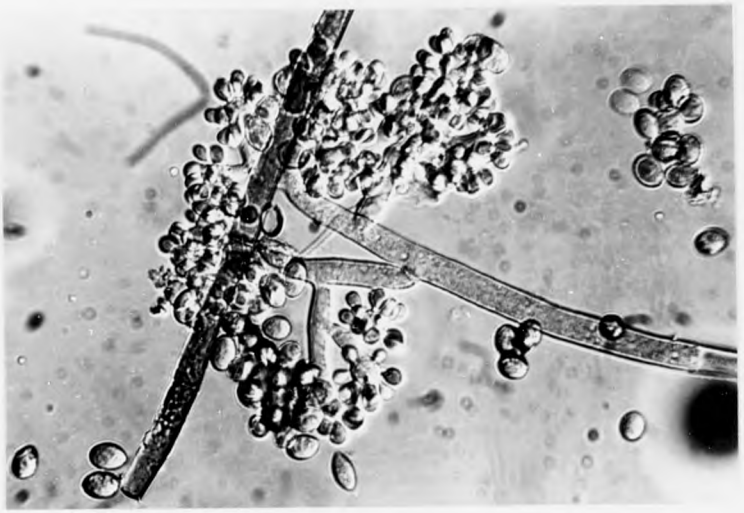
PLATE 1.2 Conidia of B. narcissicola (a) and B. cinerea (b) (x 600).

TABLE 1.2 Conidiophore branching of isolates of B. cinerea and B. narcissicola[a]

Species	Source	Number isolates examined	Number isolates with branched conidiophores: At base	At middle	At tip	Anywhere
B. cinerea	Various	13	4	12	10	13
B. cinerea	Narcissus	13	4	13	11	13
B. narcissicola	Narcissus	13	2	5	10	11

[a] Isolates grown on Medium X for 10 days at 18°C, with UV illumination.

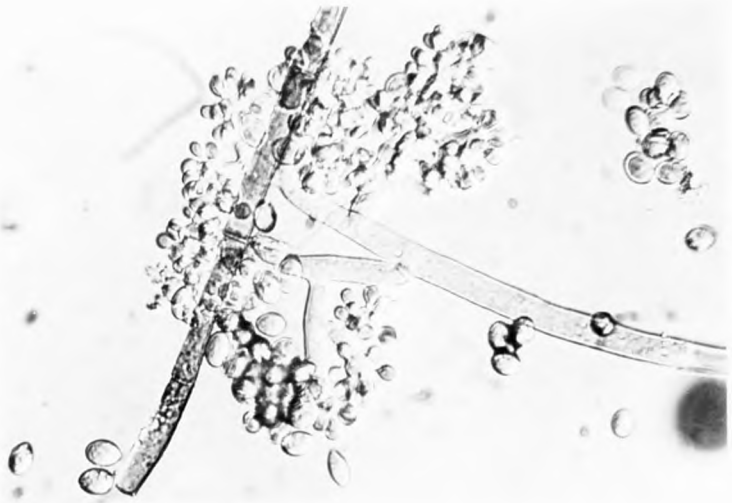
a.



b.

PLATE 1.3 Conidiophores of B. narcissicola (a) and B. cinerea (b) (x 600).

a.



b.

PLATE 1.2 Conidiophores of B. narcissicola (a) and B. cinerea (b) (x 600).

TABLE 1.3 *Pathogenicity of* B. narcissicola *and* B. cinerea *towards detached narcissus tissue, cv. Golden Harvest*

Tissue	Species	Inoculum	% inoculations in each category [a]		
			No lesion	Limited lesion	Spreading lesion
Bulb scales[b]	*B. cinerea*	conidia	60	40	0
		mycelium	97	0	3
	B. narcissicola	conidia	23	77	0
		mycelium	0	7	93
Leaves[c]	*B. cinerea*	conidia	90	10	0
		mycelium	60	40	0
	B. narcissicola	conidia	75	25	0
		mycelium	0	20	80

[a] Recorded 5 days after inoculation; mean results of five tests.

[b] Tested in October 1978.

[c] Tested in March 1979.

conidia frequently developed yellow-brown flecks after c. 1 day and within 3 days these had often coalesced to a well-defined lesion. The majority of lesions were restricted to the inoculation site (limited lesions) but a few turned a dark brown-black colour and started to spread (Plate 1.4). Inoculation sites with either no symptoms or a limited lesion when recorded after 5 days rarely spread after prolonged incubation. Spreading lesions developed more frequently at sites inoculated with B. narcissicola (6% of total sites) than with B. cinerea (< 1%). On detached leaves the majority of conidial inocula failed to cause either limited or spreading lesions. Grey flecks (water soaking) developed at some sites, particularly B. narcissicola, and occasionally progressed to form spreading lesions.

Mycelial inocula of B. narcissicola commonly caused yellow-brown flecks within 1 day of inoculation onto bulb scales. The symptoms usually developed rapidly into dark brown spreading lesions (Plate 1.5). By contrast, inoculation with B. cinerea mycelium sometimes resulted in the formation of fleck lesions but rarely caused spreading lesions. Similarly on leaves, most B. narcissicola incoula gave rise to symptoms (water soaking and grey-brown flecks) within 2 days of inoculation, which developed into spreading lesions within 5 days (Plate 1.5), whereas B. cinerea inocula caused no macroscopic symptoms (60%) or limited (40%) but not spreading lesions (Table 1.3).

B. cinerea isolates collected from narcissus caused no more lesions, either limited or spreading, than isolates collected from other host plants (Table 1.4). Two

PLATE 1.4 Pathogenicity test on narcissus bulb scales, cv. Golden Harvest, with conidial inocula of *B. cinerea*, *B. fabae*, *B. tulipae* and *B. narcissicola*; photographed 30 days after inoculation. Each scale on the upper row was inoculated with *B. cinerea* (top), *B. fabae* (left) and *B. tulipae* (right). Scales on the lower row were inoculated with three isolates of *B. narcissicola*. Note the well-defined limited lesions resulting from inoculation with *B. cinerea* and the occasional spreading lesions caused by *B. narcissicola*.

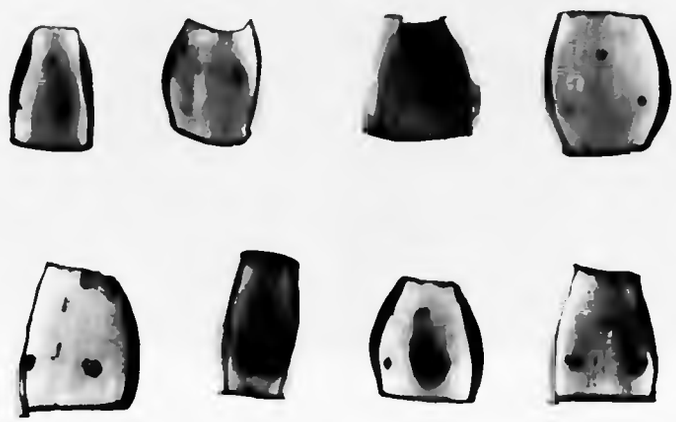
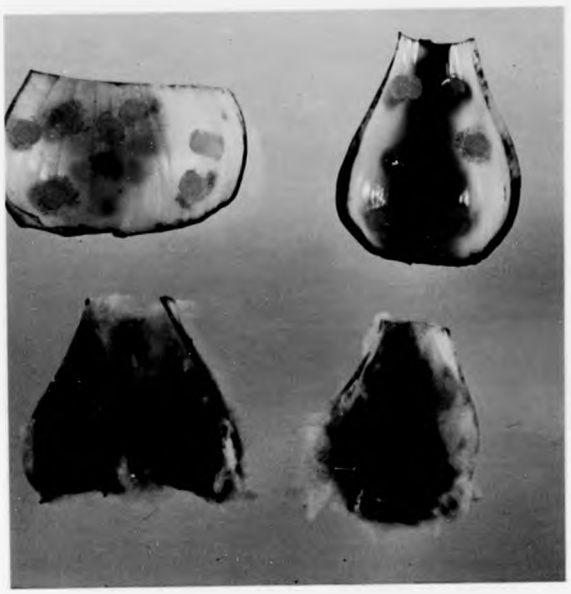


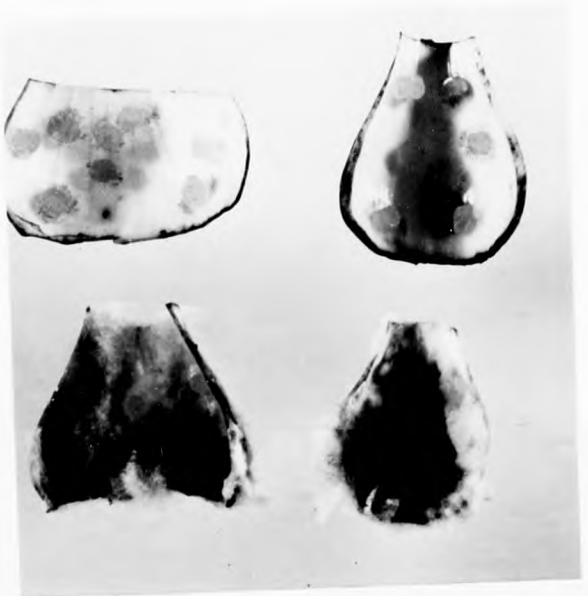
PLATE 1.4 Pathogenicity test on narcissus bulb scales, cv. Golden Harvest, with conidial inocula of *B. cinerea*, *B. fabae*, *B. tulipae* and *B. narcissicola*; photographed 30 days after inoculation. Each scale on the upper row was inoculated with *B. cinerea* (top), *B. fabae* (left) and *B. tulipae* (right). Scales on the lower row were inoculated with three isolates of *B. narcissicola*. Note the well-defined limited lesions resulting from inoculation with *B. cinerea* and the occasional spreading lesions caused by *B. narcissicola*.



B. cinerea

B. narcissicola

PLATE 1.5 Pathogenicity tests on detached bulb scales of narcissus, cv. Golden Harvest, with mycelial inocula of B. cinerea and B. narcissicola; photographed 5 days after inoculation. Note that most B. narcissicola inocula have caused spreading lesions.



B. cinerea

B. narcissicola

PLATE 1.5 Pathogenicity tests on detached bulb scales of narcissus, cv. Golden Harvest, with mycelial inocula of B. cinerea and B. narcissicola; photographed 5 days after inoculation. Note that most B. narcissicola inocula have caused spreading lesions.

TABLE 1.4 Summary of pathogenicity tests with isolates of B. narcissicola and B. cinerea on detached narcissus tissue

Tissue	Species	Source	Inoculum	Number of isolates tested	Isolate pathogenicity[a]		
					Weak	Intermediate	Strong
Bulb scales	B. narcissicola	narcissus	conidia	15	10	5	0
			mycelium	24	0	0	24
	B. cinerea	narcissus	conidia	2	2	0	0
			mycelium	11	10	1	0
	B. cinerea	various	mycelium	19	12	5	2
Leaves	B. narcissicola	narcissus	conidia	6	4	2	0
			mycelium	12	1	5	6
	B. cinerea	narcissus	conidia	2	2	0	0
			mycelium	3	3	0	0
	B. cinerea	various	mycelium	8	7	1	0

[a] Pathogenicity assessed from the number of spreading lesions formed after incubation for 5 days. Isolates causing < 10% spreading lesions were classified as weak and those causing >50% as strong.

isolates of *B. cinerea*, from field bean and from carrot, caused >50% spreading lesions in tests with mycelial inocula. By contrast, isolates of *B. narcissicola* differed only slightly in their pathogenicity towards narcissus; with mycelial inocula on bulb scales all 24 *B. narcissicola* isolates tested consistently caused >80% spreading lesions.

In summary, only mycelial inocula of *B. narcissicola* isolates consistently caused spreading lesions at the majority (>50%) of sites within 5 days of inoculation (Table 1.4). Conidial inocula of some *B. narcissicola* isolates caused >10% but < 50% spreading lesions while both conidial and mycelial inocula of *B. cinerea* isolates usually caused < 10% spreading lesions. The difference in pathogenicity towards narcissus of *B. narcissicola* and *B. cinerea* was most evident in tests with mycelial inocula on bulb scales.

Sclerotial morphology on PDA and pathogenicity tests with mycelial inocula on bulb scales were subsequently used routinely to distinguish between *B. narcissicola* and *B. cinerea*.

2. Association of B. narcissicola and B. cinerea with smoulder symptoms

The frequency with which *B. narcissicola* and *B. cinerea* are associated with typical smoulder symptoms was investigated to help define the relative roles of the two species in causing the smoulder syndrome. Samples of tissue with various symptoms were collected from commercial fields

(Kincardineshire and Lincolnshire), gauzehouse virus-tested (VT) stocks (SHRI, Dundee and ESCA, Edinburgh) and an experimental plot of infected plants (SHRI, Dundee) and examined for the presence of B. narcissicola and/or B. cinerea by isolation. Full details of the symptoms observed at different sites and times during the growing season and the corresponding isolation results are given in Chapter 2. Here, a summary is presented of the combined results.

A. Symptoms

The characteristic smoulder sympton observed in foliage was a dark brown-black lesion. This symptom was particularly common at the tips of leaves which were often fused (Plate 1.6). Similar lesions, though grey in colour, were found on flower buds (Plate 1.7). When lesions were present at the leaf base the tissue above was yellow and withered. Another common symptom was leaves curved into a sickle shape with a rot along the inner margin (Plate 1.8). In bulbs, botrytis sclerotia occurred in the outer, papery scales (Plate 1.9).

B. Isolation

The frequencies of isolation of B. narcissicola and B. cinerea from narcissus, according to symptoms, are presented in Table 1.5. B. narcissicola was the species more commonly isolated (usually >90% of isolates) from typical smoulder symptoms. B. cinerea was isolated infrequently (< 15%) from leaf tip, leaf base and flower bud lesions and was more commonly isolated from dying or dead tissue. Thirty-nine per cent of sclerotia from outer bulb scales

PLATE 1.6 Smoulder primary infection symptoms - leaf tip lesions. Shoot infection in (a) resulted from placing sclerotial infested debris in the soil c. 2cm above the nose of a healthy, virus-tested bulb. The infected shoots in (b) were produced by a bulb selected from a stock with a history of smoulder; note the fused leaf tips (arrowed).

ymptoms - leaf
sulted from
e soil c. 2cm
d bulb. The
a bulb
smoulder;

a.

b.

PLATE 1.6

PLATE 1.7 Smoulder primary infection symptoms - flower bud lesion (a) and leaf tip lesion (b). Note the leaf distortion in (b).

n symptoms - flower

). Note the leaf

a.

b.

PLATE 1.7

a.

b.

PLATE I.7

PLATE 1.8 Smoulder primary infection symptom - sickle-shaped leaf with a rot along the inner margin; note the sclerotium (arrowed).

PLATE 1.9 Sclerotia in the outer, papery scales of bulbs. Note the brown lesions in the white, fleshy bulb tissue (right).

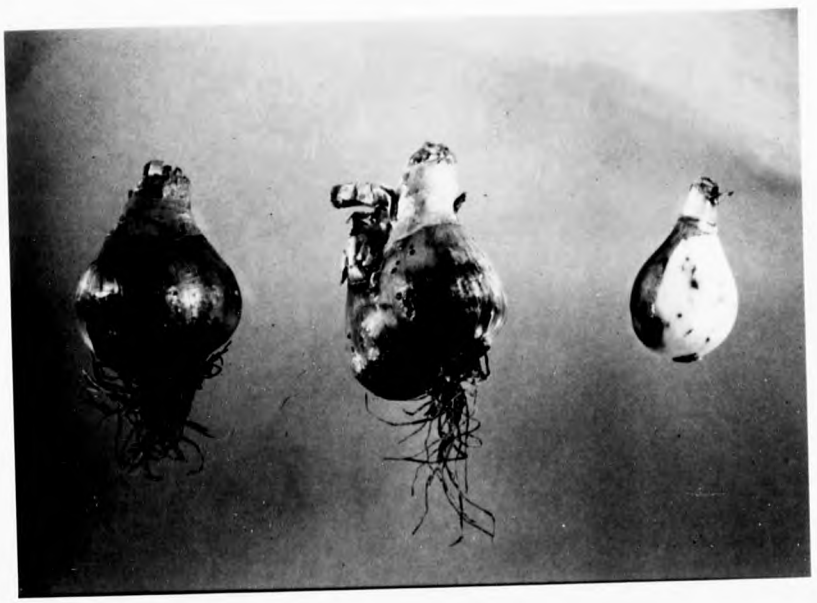


PLATE 1.9 Sclerotia in the outer, papery scales of bulbs. Note the brown lesions in the white, fleshy bulb tissue (right).

TABLE 1.5 *Isolation of* B. narcissicola *and* B. cinerea *from narcissus*

Symptom	Number of plants sampled	% samples yielding *Botrytis*	% *Botrytis* isolates identified as *B. cinerea*	*B. narcissicola*
Leaf tip lesion	57	52	10	90
Fused leaves	27	89	0	100
Leaf base lesion	50	76	3	97
Sickled leaf lesion	37	70	0	100
Healthy leaves	15	13	0	100
Senescent leaves	53	72	53	47
Flower bud lesion	14	57	12	88
Sclerotia from bulb	38	39	47	53

were germinated and isolates were identified as *B. narcissicola* and *B. cinerea* in approximately equal numbers.

The results presented here and the results of pathogenicity tests described earlier both demonstrate that *B. narcissicola* is the major cause of smoulder.

3. *The specificity of* Botrytis *species towards narcissus*

To obtain a measure of the degree of adaptation towards narcissus shown by isolates of *B. narcissicola* and *B. cinerea*, the tests described above (Section 1.1B) were extended to isolates of four other host-specific *Botrytis* species - *B. allii*, *B. elliptica*, *B. fabae* and *B. tulipae*.

Most species were tested at least twice (March and April 1978) using conidial and mycelial inocula on detached leaves and bulb scales. The percentage of inoculations causing spreading lesions, limited lesions or no symptoms 5 days after inoculation are given in Table 1.6. Only *B. narcissicola* mycelium consistently caused spreading lesions. Mycelial inocula of *B. cinerea* (8%) and *B. tulipae* (16%) gave rise to some spreading lesions in bulb tissue and the latter caused spreading lesions at 40% of inoculation sites on leaves (one test only, in May). No spreading lesions resulted from inoculation with *B. allii*, *B. elliptica* or *B. fabae*; these species gave rise to few, if any, symptoms. With conidial inoculations all six species of *Botrytis* failed to cause spreading lesions at more than 10% of the incoluation sites. Limited lesions were most frequent with *B. cinerea*, *B. narcissicola*, and *B. tulipae*. However, while the limited lesions in bulb scales caused by

TABLE 1.6 *Pathogenicity of six* Botrytis *species towards detached narcissus tissue*

Tissue	Inoculum	Species	% inoculations in each category[a]		
			No lesion	Limited lesion	Spreading lesion
Bulb scales	conidia	*B. allii*	71	29	0
		B. cinerea	60	39	1
		B. elliptica	73	27	0
		B. fabae	79	21	0
		B. narcissicola	53	41	6
		B. tulipae	57	43	0
	mycelium	*B. allii*	100	0	0
		B. cinerea	64	28	0
		B. elliptica	100	0	0
		B. fabae	100	0	0
		B. narcissicola	4	2	94
		B. tulipae	70	14	16
Leaves	conidia	*B. allii*	80	20	0
		B. cinerea	90	10	0
		B. elliptica	100	0	0
		B. fabae	91	9	0
		B. narcissicola	75	25	0
		B. tulipae[b]	90	10	0
	mycelium	*B. allii*	100	0	0
		B. cinerea	73	27	0
		B. elliptica	100	0	0
		B. fabae	100	0	0
		B. narcissicola	7	20	73
		B. tulipae[b]	20	40	40

[a] Lesions recorded 5 days after inoculation; mean results of at least two tests (March and April 1978).

[b] Tested once only, in May 1978.

B. cinerea and B. tulipae remained yellow-brown those caused by B. narcissicola often became dark brown and occasionally spread from the inoculation site. B. narcissicola would appear to be specifically adapted to invade narcissus.

4. Conditions influencing infection of narcissus by species of Botrytis

A. Age of host tissue

(i) Leaf. Narcissus leaves grow from a basal meristem so that oldest tissue is at the blade tip. Routine pathogenicity tests were carried out on the adaxial leaf surface with treatments distributed along the blade length. To examine the effect of inoculation site on lesion development, upper (tip) and lower (base) leaf halves, cv. Golden Harvest, were inoculated with either conidia or mycelium of both B. cinerea and B. narcissicola (6/4/78). Limited lesions developed at the same frequency on lower and upper leaf halves (36% of total inoculations) while spreading lesions were only slightly more common on lower (35%) than upper leaf halves (29%) (Table 1.7).

Leaves of field grown narcissus emerged at Stirling during January and February, reached maturity (full size) in March and April and remained green until July. To investigate the effect of leaf age on lesion development pathogenicity tests were carried out at monthly intervals from March to June. Leaves of cv. Golden Harvest were inoculated with conidia and mycelium of six Botrytis species (B. allii, B. cinerea, B. elliptica, B. fabae, B. narcissicola and B. tulipae) and the percentages of spreading lesions were recorded after 5 days (Table 1.8). A change in tissue

TABLE 1.7 Infection of upper and lower leaf halves by B. cinerea and B. narcissicola

Inoculum	Species	% inoculations in each category [a]					
		No lesion		Limited lesion		Spreading lesion	
		Upper	Lower	Upper	Lower	Upper	Lower
Conidia	B. cinerea	85	75	15	25	0	0
	B. narcissicola	50	45	50	55	0	0
Mycelium	B. cinerea	100	70	0	30	0	0
	B. narcissicola	2	2	48	37	50	61
	Total[c]	35	28	36	37	29	35

[a] Lesions recorded 5 days after inoculation; 20 inocula and five leaf halves per treatment.

[b] Pooled results for four isolates (80 inocula).

[c] Percentage of total inocula on upper or lower leaf halves falling into each of the three categories.

TABLE 1.8 Influence of leaf senescence on the pathogenicity of six Botrytis species towards narcissus leaves

Inoculum	Species	% inocula causing spreading lesions[a]			
		March	April	May	June
Conidia	*B. allii*	0	-[d]	-	0
	B. cinerea[b]	0	0	0	0
	B. elliptica	0	-	-	0
	B. fabae	0	0	0	0
	B. narcissicola[c]	0	0	0	30
	B. tulipae	0	-	0	10
Mycelium	*B. allii*	0	-	-	0
	B. cinerea[b]	0	0	0	0
	B. elliptica	0	-	-	0
	B. fabae	0	0	0	0
	B. narcissicola[c]	70	56	85	95
	B. tulipae	0	-	40	0

[a] Recorded 5 days after inoculation; 10 inocula per treatment distributed over 10 leaves.

[b] Mean results of two isolates.

[c] Mean results of three isolates.

[d] Not tested.

susceptibility with age was most noticeable using conidial inocula. No lesions, either limited or spreading, resulted in March or April. In May, occasional grey flecks (water soaking) occurred at B. narcissicola inoculation sites. On leaves tested in June, as the first signs of senescence were appearing, B. cinerea, B. narcissicola and B. tulipae all gave rise to occasional grey fleck lesions within 2 days of inoculation and the latter two species had resulted in some spreading lesions (30% and 10%) after 5 days. Mycelial inocula of B. narcissicola generally caused a high percentage of spreading lesions at all times. B. allii, B. cinerea, B. elliptica and B. fabae all failed to cause spreading lesions, even in June, and B. tulipae did so in only one test (May).

(ii) Bulb. The narcissus bulb is a complex structure comprising scales, leaf bases, flower stalk bases and inflorescences (buds); a full description of bulb morphology is given in Appendix 1. Routine pathogenicity tests were carried out on a range of fleshy bulb scales and leaf bases of second generation bulb tissue. To investigate possible variation in lesion development according to the age or type of bulb tissue, pathogenicity tests with B. narcissicola mycelial inocula were performed on identified tissues dissected from single-nose (10-12cm diameter) bulbs, cv. Golden Harvest. The percentage of spreading lesions recorded 5 days after inoculation are given in Table 1.9. Variation between bulbs was large but some differences according to tissue type were apparent. The least number of spreading lesions developed in the thick flower stalk base (OFS2) and semi-sheathing leaf base (LB2a) of second generation tissues and the thin, outer scale (LB3b), of third generation

TABLE 1.9 Infection of bulb tissues by B. narcissicola

Bulb unit generation	Tissue[a]	Total number of inoculations	% inoculations causing spreading lesions[b] Per tissue	Per generation
First	TBS1a	30	77 (67-100)	
	TBS1b	30	67 (33-83)	76 (67-83)
	TBS1c	30	83 (53-100)	
Second	OFS2	20	45 (0-75)	
	LB2a	20	40 (0-50)	
	LB2b*	40	73 (38-100)	
	LB2c*	40	53 (25-63)	57 (40-70)
	S2a*	40	65 (25-100)	
	S2b*	40	55 (25-100)	
	S2c*	40	70 (25-88)	
Third	LB3a	30	75 (37-100)	
	LB3b	30	31 (13-50)	53 (31-75)

[a] Bulb structure and the tissue code are described in Appendix 1.

[b] Mean and range of means (min.-max.) for 5 bulbs.

* Used in routine pathogenicity tests.

tissues. Bulb scales of the first generation, surrounding the terminal bud, were most susceptible. Similar numbers of spreading lesions (53-73%) were produced in the different bulb scale and leaf base tissues of the second generation unit used in routine pathogenicity tests.

To investigate possible changes in the susceptibility of bulb tissue during shoot growth, selected bulbs of cv. Golden Harvest (single nose, 10-12cm diameter) were planted outside (29/9/78) and at five c. 1 month intervals two bulbs were removed and the fleshy second generation bulb tissues inoculated with B. narcissicola mycelium. The rates of lesion spread in bulb tissue increased during the growing season (Fig. 1.2), although in all five tests the percentage of spreading lesions recorded after 5 days was high (Table 1.10).

The effect of prolonged bulb storage on susceptibility to infection from Botrytis was also investigated. Lesion development was compared in bulbs stored at $4^{o}C$ for increasing length of time with lesion development in bulbs tested immediately after lifting (August). No consistent differences in susceptibility were apparent, from either conidial or mycelial inocula, of six Botrytis species, tested at 3 month intervals for 1 year (Table 1.11).

B. Wounds

The effect of wounds, often reported as a factor enhancing the ability of B. cinerea to colonise tissue (Jarvis, 1977) was investigated by wounding narcissus leaf and bulb tissue prior to inoculation with conidia of B. narcissicola and B. cinerea.

FIG. 1.2 Change in the rate of lesion spread in bulb tissue (cv. Golden Harvest) with shoot growth and development. Single nose bulbs (10-12cm diameter) were planted on 29/9/78 and at c. 1 month intervals two bulbs were dug up and bulb scales and leaf bases enclosing the emerging shoot (second generation tissues) were inoculated with mycelium of B. narcissicola. A minimum of 60 inocula were used in each test. Bulbs were tested on 5/10/78 (□—□), 27/10/78 (○—○), 24/11/78 (■—■), 17/12/78 (●—●) and 25/1/79 (▲—▲). The mean lesion diameter attained after 5 days was significantly greater ($P < 0.05$) in the last test than the first.

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FIG. 1.2

TABLE 1.10 Influence of development after planting on the pathogenicity of B. narcissicola towards bulb tissue

Date bulbs lifted[a]	Shoot length (cm)	Number of inocula per isolate	% spreading lesions for three isolates[b] 1 (11A)	2 (D4)	3 (527)
5/10/78	0	20	90	45	55
24/10/78	0	10	100	50	70
24/11/78	1-3	45	84	91	91
17/12/78	5-7	45	-	82	96
25/1/79	7-9	45	100	96	98

[a] Single nose bulbs, cv. Golden Harvest, planted in August 1978.

[b] Recorded 4 days after inoculation with mycelium; mean results for 2 bulbs.

TABLE 1.11 Influence of bulb storage at 4°C on the infection of detached bulb scales by six species of Botrytis

Inoculum	Species	Infection of bulbs stored for: [a] 0	3	6	9	12 months
Conidia	B. allii	-[d]	0	0	0	0
	B. cinerea[b]	0	0	4 (0-8)	0	4 (0-8)
	B. elliptica	-	0	0	0	-
	B. fabae	0	0	0	0	0
	B. narcissicola[c]	9 (0-23)	2 (0-3)	13 (0-20)	9 (0-20)	12 (10-14)
	B. tulipae	-	0	0	0	-
Mycelium	B. allii	-	-	0	0	-
	B. cinerea[c]	0	0	5 (0-20)	0	-
	B. elliptica	-	0	0	0	-
	B. fabae	0	0	0	0	-
	B. narcissicola[c]	100	100	100	100	-
	B. tulipae	-	15	16	8	-

[a] % spreading lesions recorded 5 days after inoculation.

[b] Mean (and range of means) for two isolates.

[c] Mean (and range of means) for five isolates.

[d] Not tested.

(i) Leaf. Light bruising of detached leaves was insufficient to allow infection (Table 1.12). However, when the epidermis was broken, by pricking with a sterile needle, 14% of B. narcissicola conidial inocula produced spreading lesions and when the epidermis was removed this figure rose to 40%. Colonization from B. cinerea inocula (21%) occurred only after freezing a 5mm diameter disc of tissue by touching with a metal rod previously cooled in liquid nitrogen; this treatment allowed 90% of B. narcissicola inocula to spread.

(ii) Bulb. In bulb tissue, removing the epidermis or pricking with a needle again allowed invasion from some B. narcissicola conidial inocula (5 and 13% respectively) but not from B. cinerea (Table 1.13).

C. Nutrients

Infection of narcissus tissue from conidial inocula of B. narcissicola was rare and yet mycelial inocula on nutrient-rich V_8 juice agar (25% V_8 juice, v/v) regularly gave rise to spreading lesions (Section 1.1B). The effect of adding nutrients, including pollen grains, to conidial suspensions was therefore investigated. B. cinerea was also tested in each treatment to further the comparison of pathogenicities of the two species.

(i) Leaf. On detached leaves, inoculations of conidia in 10% V_8 juice (pH6) resulted in 75% spreading lesions from B. narcissicola and 5% from B. cinerea (Table 1.12). When mixed with narcissus pollen at concentration of 10^4 grains/ml or greater, B. narcissicola conidial inocula caused some spreading lesions; at 10^6 grains/ml all inocula gave rise to

TABLE 1.12 Influence of tissue damage and nutrient addition to inoculum droplets on the infection of detached leaves (cv. Golden Harvest) from conidia of B. cinerea and B. narcissicola

Treatment[a]	Infection by B. cinerea[b]				Infection by B. narcissicola[b]			
	TS	NL	LL	SL	TS	NL	LL	SL
1. Standard (conidia in SDW)								
10^5/ml	120	90	10	0	180	75	25	0
10^6/ml	20	80	20	0	20	60	40	0
2. Wounded								
Bruised	10	*	100	0	10	*	100	0
Pricked	50	75	25	0	50	76	10	14
Epidermis removed	20	50	50	0	20	45	15	40
Frozen	70	*	79	21	70	*	10	90
3. Nutrients added								
5mM glucose	20	65	35	0	20	70	30	0
50mM glucose	20	55	45	0	20	65	35	0
1nM glutamine	20	45	55	0	20	20	80	0
1mM glutamine	20	50	50	0	20	30	70	0
5mM glucose/1mM glutamine	20	85	15	0	20	30	70	0
Pollen (grains/ml) 10^3	-[c]	-	-	-	40	95	5	0
10^4	-	-	-	-	40	77	18	5
10^5	20	100	0	0	50	42	28	30
10^6	20	*	100	0	20	0	0	100
10% V8 juice, pH 6	20	0	95	5	20	10	15	75
4. Wounded and nutrients								
Pricked-Pollen (10^5/ml)	48	36	58	6	48	16	6	78
Pricked-Pollen (10^6/ml)	20	*	100	0	20	0	0	100
Frozen-Pollen (10^5/ml)	10	*	100	0	10	*	10	90

[a] Control lacking conidia were included with each treatment; no control inoculations resulted in lesion formation.

[b] Inoculation sites categorized 5 days after inoculation into no lesions (NL), limited lesions (LL) and spreading lesions (SL). Results are expressed as percentages of the total number of inoculation sites (TS) within a treatment.

[c] Not tested.

* LL-NL distinction blurred by tissue damage or added nutrients.

TABLE 1.13 Influence of tissue damage and nutrient addition to inoculum droplets on infection of detached bulb tissue (cv. Golden Harvest) from conidia of B. cinerea and B. narcissicola

Treatment[a]	Infection by B. cinerea[b]				Infection by B. narcissicola[b]			
	TS	NL	LL	SL	TS	NL	LL	SL
1. Standard (conidia in SDW)								
10^5/ml	120	60	40	o	200	52	46	2
10^6/ml	20	60	40	0	20	30	65	5
2. Wounded								
Pricked	30	33	67	0	30	17	70	13
Epidermis removed	80	22	78	0	60	27	68	5
3. Nutrients added								
5mM glucose	30	93	7	0	30	13	87	0
1nM glutamine	30	83	17	0	30	40	60	0
Czapek Dox	10	0	90	10	10	0	50	50
SPN (pH 4)	10	0	100	0	10	0	80	20
Pollen (10^5 grains/ml)	10	0	100	0	10	0	0	100
4. Wounded and nutrients								
Pricked-glucose (5mM)	30	50	50	0	30	34	43	23
Pricked-glutamine (1nM)	30	17	60	23	30	0	80	20

[a,b] As for Table 1.12.

spreading lesions. No spreading lesions resulted from B. cinerea - pollen inoculations. Addition to inocula of a simple sugar (glucose, 5mM and 50mM) and amino acid (glutamine, 1nM and 1mM), either singly or in combination, increased the frequency of limited lesions but no spreading lesions resulted. When pricking damage was combined with pollen addition (10^5 grains/ml) the percentage of spreading lesions resulting from B. narcissicola conidial inocula was greater than the sum of spreading lesions for the two treatments given independently. A small percentage of B. cinerea conidial inocula also resulted in spreading lesions, where neither pricking nor pollen alone was successful. Invasion of freeze-killed tissue from botrytis conidia was not enhanced by pollen addition.

(ii) Bulb. On bulb tissue, adding pollen grains (10^5/ml) again resulted in spreading lesions from B. narcissicola inocula but not B. cinerea, while glucose (5mM) and glutamine (1nM) had no effect on lesion development (Table 1.13). Suspensions of B. narcissicola conidia in Czapek Dox or SPN both caused some spreading lesions (Plate 1.10). No inoculations of B. cinerea conidia in SPN and only 10% in Czapek Dox caused spreading lesions.

D. Narcissus cultivar

Second generation fleshy tissue dissected from bulbs of eight cultivars was tested for its susceptibility to infection from three isolates of B. narcissicola using mycelial inocula. The experiment was performed twice. All eight cultivars were susceptible and the majority of inoculations resulted in spreading lesions.

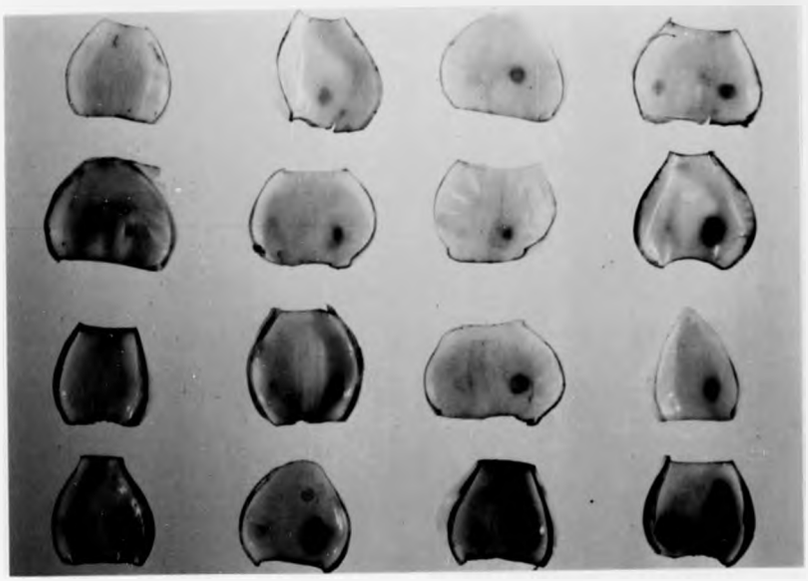

PLATE 1.10 Influence of nutrients on the infection of bulb scales by B. cinerea and B. narcissicola. Bulb scales were inoculated with conidia in SDW (top row), Czapek Dox (second row) or SPN (third row), or with mycelium on V8 juice agar (bottom row). On each scale B. cinerea inocula are on the left, B. narcissicola on the right and a control is above them. Note that all B. narcissicola mycelial inocula have caused spreading lesions whereas only one inoculum of B. narcissicola conidia in Czapek Dox or in SPN appears to be spreading. Note also that limited lesions are more evident at sites inoculated with B. narcissicola than B. cinerea conidia. Photographed 3 days after inoculation.

PLATE 1.10 Influence of nutrients on the infection of bulb scales by B. cinerea and B. narcissicola. Bulb scales were inoculated with conidia in SDW (top row), Czapek Dox (second row) or SPN (third row), or with mycelium on V8 juice agar (bottom row). On each scale B. cinerea inocula are on the left, B. narcissicola on the right and a control is above them. Note that all B. narcissicola mycelial inocula have caused spreading lesions whereas only one inoculum of B. narcissicola conidia in Czapek Dox or in SPN appears to be spreading. Note also that limited lesions are more evident at sites inoculated with B. narcissicola than B. cinerea conidia. Photographed 3 days after inoculation.

Cultivar sensitivity to B. narcissicola was investigated by recording rates of increase in lesion diameters following inoculation with mycelium. Golden Harvest was found to be the most and cvs. Dutch Master and Geranium the least sensitive (Fig. 1.3).

E. Incubation temperature

When inoculated onto undamaged leaves in SDW (24/4/78), conidia of B. narcissicola and B. cinerea caused spreading lesions at < 10% of sites at any of the temperatures tested (4, 10, 18 or 25°C). Spreading lesions developed more rapidly at 18°C than 4 or 10°C when pollen was added to inocula, (Table 1.14).

FIG. 1.3 Rates of lesion spread in bulb tissue of eight narcissus cultivars following inoculation with mycelium of B. narcissicola. Each cultivar was tested on 13/10/78 (a) and on 27/10/78 (b). Note that the rate of lesion spread is highest in cv. Golden Harvest (GH) and lowest in cvs. Dutch Master (DM) and Geranium (G). Other cultivars tested were Barrett Browning (BB), King Alfred (KA), Lothario (L), Sempre Avanti (SA) and Yellow Cheerfulness (YC).

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with mycelium
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lowest in cvs.
r cultivars tested
A), Lothario (L),
(YC).

FIG. 1.3

TABLE 1.14 Influence of incubation temperature on infection of leaves from B. cinerea and B. narcissicola conidial inoculation

Inoculum		% inoculations resulting in spreading lesions[a]			
		4°C	10°C	18°C	25°C
SDW	B. cinerea	0	0	0	0
	B. narcissicola	0	0	0	0
Pollen[b]	B. cinerea	0	0	0	0
	B. narcissicola	0	67	100	100
Pollen-prick	B. cinerea	0	3	7	-[f]
	B. narcissicola	58[c]	100[d]	100[e]	-

[a] Recorded 5 days after inoculation.

[b] 10^6 grains/ml.

[c] Recorded 10 days after inoculation; no spreading lesions at 5 days.

[d] 100% spreading lesions reached after 5 days.

[e] 100% spreading lesions reached after 3 days.

[f] Not tested.

CHAPTER 2

SMOULDER EPIDEMIOLOGY

Previous reports on the life-cycle of *B. narcissicola* and smoulder epidemiology are based more on observation than experiment. This chapter describes experiments investigating the disease cycle and factors influencing the occurrence of smoulder outbreaks.

1. Symptoms

In order to describe all symptoms which might be associated with smoulder, an experimental planting of naturally-infected narcissus (cv. Verger) and commercial field-plantings (cv. Golden Harvest) were inspected regularly throughout the growing season. Several bulb lots were also examined. The incidence of different symptoms were estimated and tissues were tested for the presence of *B. narcissicola* and/or *B. cinerea* by isolation.

A. Description

Plants in the experimental plot were examined during 1978 and 1979 at 2-3 wk intervals from foliage emergence to dieback. The symptoms observed are listed in Table 2.1 and the more common are illustrated in Plates 1.6-1.9 and 2.1-2.3. All symptoms from which *B. narcissicola* was consistently isolated (> 50% of samples) were regarded as part of the smoulder syndrome. They were classified as either primary symptoms (Plates 1.6-1.9) or secondary symptoms (Plates 2.1-2.3) according to their presence at emergence or development later in the season. Additional symptoms noted in commercial planting of cv. Golden Harvest at Cushnie Farm are also described.

In bulbs, an obvious symptom of botrytis infection was the presence of sclerotia in the outer, papery scales, which

TABLE 2.1 Symptoms observed in shoots of narcissus at SHRI (Site A, cv. Verger) and at Cushnie Farm (Site B, cv. Golden Harvest

Site	Symptom[a]	Occurrence during 1978	Probable[b] cause	Plate
A	Primary smoulder symptoms			
	1. Failure to emerge	Th.	BN	
	2. Shoot collapse		BN	
	3. Dark brown leaf tip lesion; sometimes leaves fused together		BN	1.6
	4. Leaf distortion, often near tip, sometimes with a lesion	March April	BN, (eelworm, virus)	1.7b
	5. Leaf curved into a sickle shape, often with a rot along the inner margin	May	BN, (BSM)	1.8
	6. Flower bud lesion		BN	1.7a
	Secondary smoulder symptoms			
	1. Brown spot lesion, often at leaf or flower stalk base	April	BN	2.1a
	2. Leaf base lesion, with withering above	May June	BN	2.1b
	3. Brown-black leaf streaks		BN, (virus, physiological)	2.2b
	4. Rusty-brown flecking on leaves and stalks, sometimes as streaks	June	BN	2.2a
	5. Isolated dead leaves in otherwise healthy shoots	July	BN, (senescence)	2.5
	Other symptoms			
	1. Yellow-brown leaf tips	March	Frost, wind damage	
	2. Necrotic, brown leaf tips and flower stalks	June	Senescence	
	3. Yellow-brown mottle, especially along leaf margin	July	Senescence	
	4. Grassiness	Th.	BN, BSM, BF, virus	
	5. White stripes and yellow streaks in leaves		Virus	

(contd.)

TABLE 2.1 (contd.)

Site	Symptom	Occurrence during 1978	Probable[b] cause	Plate
B	Smoulder symptoms			
	1. Lesion in the leaf sheath, often below the soil surface (primary symptom)	Th.	BN	2.4
	2. Sclerotia in rotted tissue	July	BN(BC)	2.4b
	3. Flower spotting (secondary symptom)	April	BN, BC	
	4. Flower stalk end rot (secondary symptom)	April	BN(BC)	2.3
	5. Broken leaves rotting (secondary symptom)	May June	BN(BC)	2.3b
	Other symptoms			
	1. Leaf tip chlorotic mottle	March	HWT damage	
	2. Grey spot lesion, often in the centre of the leaf blade	June	Unknown	

[a] Symptoms at Site A are listed in three groups according to their probable cause - smoulder primary symptoms, smoulder secondary symptoms and other symptoms. Symptoms described for site B are additional to those described for site A.

[b] Based on isolation data (see Tables 2.2 and 2.3). Terms in parentheses refer to alternative agents possibly causing similar symptoms.

Abbreviations: BN, Botrytis narcissicola; BC, Botrytis cinerea; BF, bulb flies; BSM, Bulb scale mite; HWT, hot water treatment; Th., throughout.

PLATE 2.1 Smoulder secondary infection symptoms;
(a), dark-brown spot lesion, near the base of a leaf;
(b), dark-brown leaf base lesion, with withering above.

ection symptoms;

the base of a leaf;

with withering above.

a.

b.

PLATE 2.1

a.

b.

PLATE 2.1

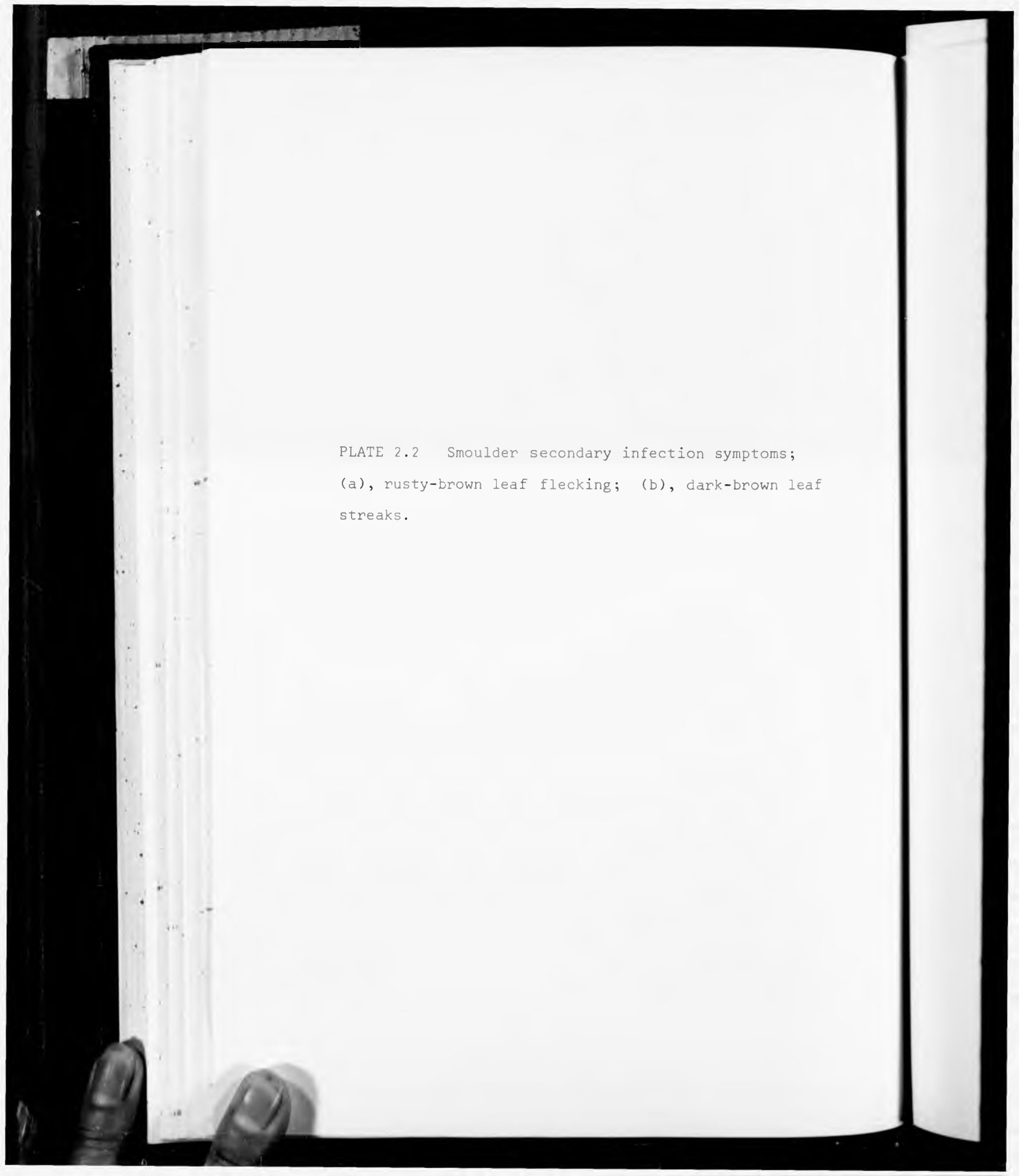
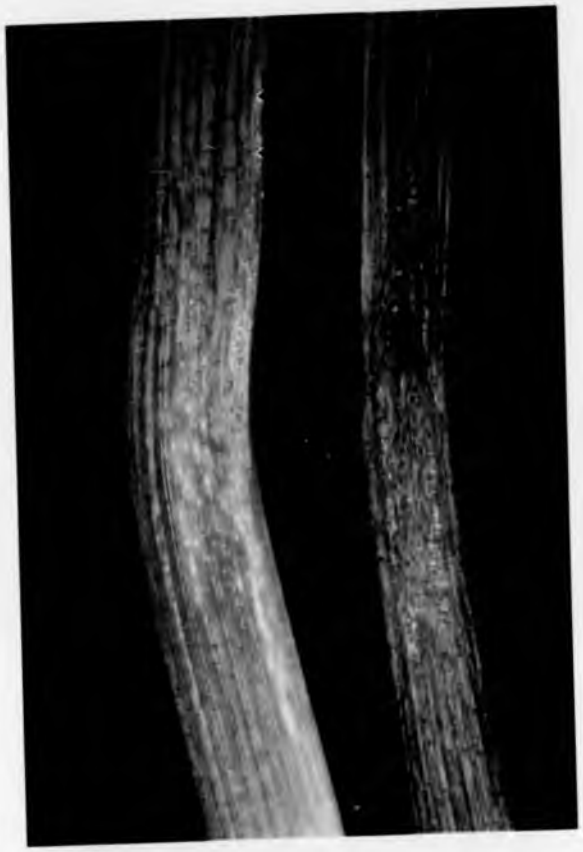


PLATE 2.2 Smoulder secondary infection symptoms; (a), rusty-brown leaf flecking; (b), dark-brown leaf streaks.

ection symptoms;

), dark-brown leaf

a.



b.

PLATE 2.2

a.

b.

PLATE 2.3 Smoulder secondary infection symptoms; (a), a rotting flower stalk with a lesion in the adjacent leaf (behind); rusty-brown leaf flecking is also present (right); (b) dark-brown lesions in three flower stalks and a broken leaf, after flower picking. In both (a) and (b) note the sporulating mycelium.

a.

b.

PLATE 7.3 Shoulder secondary infection symptoms: (a). A rotting flower stalk with a lesion in the adjacent leaf (bottom); rusty-brown leaf flecking is also present (right); (b) rust-brown lesions in three flower stalks and a bract (top left, after flower picking. In both (a) and (b) note the [illegible] mycelium.

were usually light brown but occasionally a darker, chocolate brown colour. Diffuse grey patches and small brown lesions were occasionally present in the outermost fleshy scale; the latter were often located beneath sclerotia (see Plate 1.9).

B. Isolation of B. narcissicola and B. cinerea

The frequencies of isolation of B. narcissicola and B. cinerea from shoot and bulb tissues, both with symptoms and apparently healthy, are presented in Tables 2.2 and 2.3. B. narcissicola was isolated most frequently (>50% of samples) from the dark brown lesions in leaves, leaf sheaths and flower buds (Table 2.2). Leaves curved into a sickle shape but without a lesion along the inner edge occasionally (30% of samples) yielded B. narcissicola. B. cinerea was isolated, albeit infrequently, from lesions at the leaf tip (8%), the leaf base (2%), in the leaf sheath (3%) or in flower buds (8%).

Botrytis narcissicola was isolated frequently from leaves with rusty-coloured flecks (82%) and black-brown streaks (50%) and occasionally from grey spot lesions (19%). Botrytis cinerea was also isolated from grey spot lesions (22%) and once from a black-brown streak. Neither species was isolated from yellow or pale brown leaf tips or the white fleck symptom.

Towards the end of the growing season, both B. narcissicola and B. cinerea were commonly isolated from leaf and flower stalk debris, but only B. narcissicola was isolated from spreading lesions in otherwise healthy flower stalks and broken leaves.

TABLE 2.2 Isolation of B. narcissicola and B. cinerea from narcissus shoots.

Symptom	Isolation date	Site	Sample size	Samples yielding[a] B. narcissicola Number	%	B. cinerea Number	%
Healthy tissue							
1. Sheath (bulb scale leaf)	3/79	Cushnie	34	6	18	1	3
2. Leaf tip	5/78	SHRI	8	0	0	0	0
3. Leaf tip	3/79	Cushnie	30	8	27	1	3
4. Leaf mid	3/79	Cushnie	15	2	13	0	0
5. Leaf base	3/79	Cushnie	15	2	13	0	0
6. Flower bud	3/79	Cushnie	27	1	4	0	0
7. Leaf	6/80	Cushnie	30	2	6	1	3
8. Leaf	6/80	SHRI	30	0	0	0	0
9. Flower stalk	6/80	Cushnie	18	3	17	1	6
Flecks and spots							
1. Yellow leaf tip, edge	5/78	SHRI	7	0	0	0	0
2. Pale brown leaf tip	6/79	Cushnie	10	0	0	0	0
3. Rusty brown leaf and stem fleck	6/79	Cushnie	22	18	82	0	0
4. Rusty brown leaf and stem fleck	6/80	Cushnie	22	10	45	2	10
5. Grey leaf spot	6/79	Cushnie	27	5	19	6	22
6. Grey leaf spot	6/80	Cushnie	20	2	10	1	5
7. Brown leaf spot	5/78	SHRI	3	2	67	1	33
8. White leaf fleck	6/80	Cushnie	30	1	3	0	0
9. Flower spot	5/79	Cushnie	10	4	40	0	0
Lesions							
1. Sheath	3/79	Cushnie	120	69	58	3	3
2. Leaf tip	5/78	SHRI	11	7	64	3	27
3. Leaf tip	3/79	Cushnie	45	21	47	2	4
4. Leaf tip	4/79	ESCA	5	4	80	1	20
5. Fused leaf tips	3/79	Cushnie	18	17	94	0	0
6. Leaf base	6/79	Cushnie	50	37	74	1	2
7. Sickled leaf	5/78	SHRI	5	5	100	0	0
8. Sickled leaf	3/79	Cushnie	43	33	77	0	0
9. Sickled leaf	4/79	ESCA	5	5	100	0	0

(contd.)

TABLE 2.2 (contd.)

Symptom	Isolation date	Site	Sample size	Samples yielding[a] B. narcissicola Number	%	B. cinerea Number	%
10. Sickled leaf (no lesion)	3/79	Cushnie	23	7	30	0	0
11. Shoot collapse	3/79	Cushnie	10	8	80	0	0
12. Flower bud	3/79	Cushnie	12	6	50	1	8
Wounded tissue							
1. Flower stalk	5/79	Cushnie	50	34	68	0	0
2. Broken leaf	5/79	Cushnie	10	8	80	0	0
Dead tissue							
1. Leaf	5/78	SHRI	41	26	63	14	34
2. Leaf	6/80	Cushnie	10	5	50	2	20
3. Flower stalk	6/78	SHRI	6	3	50	2	33
4. Flower head	6/78	SHRI	7	0	0	7	100
5. Flower head	6/79	Cushnie	10	10	100	0	0

[a] Identified from sclerotia produced on PDA.

TABLE 2.3 *Isolation of* B. narcissicola *and* B. cinerea *from narcissus bulbs, cvs. Golden Harvest and Sempre Avanti*

Symptom	Sample size	Samples yielding[b] B. narcissicola		B. cinerea	
		Number	%	Number	%
Outer papery scale					
Light brown	232	23	10	0	0
Dark brown	86	19	22	0	0
Sclerotia	38	8	21	7	18
Inner fleshy scale					
Healthy	26	0	0	0	0
Grey patch	26	0	0	0	0
Brown lesion	15	1	7	0	0

[a] Bulb obtained from Grampian Growers Ltd., Montrose; examined in October 1978.

[b] Identified from sclerotia produced on PDA.

The planting site from which samples were collected influenced the isolation results (Table 2.2). On dead flower heads only B. cinerea was found at SHRI and only B. narcissicola at Cushnie Farm.

Results of isolations from bulbs are given in Table 2.3. Thirty-nine per cent of sclerotia removed from outer bulb scales were successfully germinated; of these, 47% were identified as B. cinerea and 53% as B. narcissicola. Where there were no sclerotia, B. cinerea was not isolated from papery scales but B. narcissicola was isolated from both chocolate brown scales (22%) and light brown scales (10%). No botrytis isolates were obtained from white, fleshy scales or from grey patches in them and B. narcissicola was isolated from only one of 15 brown lesions (Table 2.3).

C. Incidence

(i) Shoot symptoms in commercial plantings. Estimates of the incidence of smoulder were made in naturally infected plantings of narcissus, cv. Golden Harvest at Cushnie Farm, in March and June 1979. Because of the difficulty in distinguishing between adjacent plants (bulb clusters), the incidence of symptoms was recorded on the basis of individual shoots. Estimates were obtained for each symptom and for a total incidence of smoulder (summation of symptoms) within a planting (Tables 2.4 and 2.5). Symptoms recorded in March (dark brown lesions and collapsed shoots) were considered to be primaries and those recorded in June (flecks, streaks, spots and leaf base lesions) were taken as symptoms of secondary infection.

TABLE 2.4 The incidence of smoulder primary infection symptoms in commercial plantings of narcissus, cv. Golden Harvest, at Cushnie Farm (28/3/79)

Age of planting (yrs)	Mean % of shoots with symptoms[a]					Mean number of shoots/m
	Leaf sheath lesion	Leaf tip lesion	Sickled leaf lesion	Collapsed shoot	Any symptom[b] on leaf	
1	-[c]	0.25	0	0	0.25	32.1
3	1.05	1.16	0.43	0.36	1.95	55.2

[a] Estimated from five, randomly selected 10m lengths of ridge; each shoot placed in one symptom class only.

[b] Excluding leaf sheath lesions.

[c] Leaf sheaths not visible.

TABLE 2.5 The incidence of smoulder secondary infection symptoms in commercial plantings of narcissus cv. Golden Harvest, at Cushnie Farm, (6/6/79).

Age of planting (yrs)	Mean % of shoots with symptoms[a]								
	Brown fleck	Black streak	Grey Spot	Leaf tip lesion [b]	Leaf base lesion [b]	Leaf edge lesion [b]	Dead Leaf	Total	Number of shoots/m
1	2.80	1.80	2.90	1.90	0.20	0.40	1.10	3.60	25.8
3	14.0[c]		9.50	3.50	7.18	3.23	1.40	15.31	43.3

[a] Estimated from five, randomly selected, 10m lengths of ridge; each shoot placed in one symptom class only.

[b] Only one leaf per shoot with the symptom (i.e. not a late primary).

[c] Flecks and streaks combined.

Smoulder primaries (1.95% of shoots) were more prevalent in the shoots of bulbs planted in autumn 1976 (third season in the ground) than in the shoots of bulbs planted in autumn 1978 (0.25%). Leaf tip lesions were the most common symptom. Secondary infection symptoms recorded in June were also more frequent in the three year old planting; common symptoms were rusty-brown flecks and streaks (14.1%), grey spot lesions (9.5%) and leaf base lesions (7.2%). Interestingly, rots developed in the ends of most flower stalks after picking and also in leaves broken during picking.

(ii) Shoot symptoms in an experimental planting recorded during two seasons. In the plot of Verger at SHRI a lower planting rate allowed discrimination between shoots of adjacent bulb clusters. During 1978 and 1979 the planting was examined at c. 1 month intervals, from shoot emergence (February/March) to shoot death (July), and the incidence of smoulder scored on a bulb cluster basis. Additionally in 1979, individual shoots were scored for smoulder and leaf numbers counted (Tables 2.6 and 2.7).

Comparing the results of 1978 and 1979, several differences in symptom frequency were apparent. In particular, the proportion of shoots emerging with primary smoulder symptoms was much greater in the second year. Clusters with grassy shoots were also more frequent in 1979 (10-15%) than 1978 (1%).

Leaf tip lesions were the most common symptom each year. In 1978 the incidence rose from 1.6% of clusters in March to 5.7% in May, and then declined as leaves died

TABLE 2.6 The incidence of smoulder symptoms (per cluster) in an experimental planting of narcissus, cv. Verger, during 1978 and 1979

Symptom[a]	% clusters with symptom at inspection[b]											
	1978 (first season)							1979 (second season)				
	20/3	11/4	2/5	17/5	7/6	21/6	5/7	3/4	20/4	7/5	28/5	22/6
No shoot emergence	1.0	1.0	-	-	-	-	-	3.7	2.1	2.1	-	-
Leaf tip distortion	0	0.8	1.8	2.3	1.6	0.2	0.2	5.5	12.0	18.1	15.4	-
Leaf tip lesion	1.6	2.7	4.0	5.7	5.5	2.3	0	19.0	26.5	31.8	37.7	36.2
Sickled leaf with lesion	0	0.4	0.6	2.5	1.9	0	1.3	2.7	4.0	6.1	2.9	-
Sickled leaf, no lesion	0	0	0.4	0.2	0.4	0	0	4.2	2.1	3.5	1.7	-
Collapsed shoot	0	0.4	0.4	0.5	0.2	0	0	3.4	0.4	0.8	1.5	2.5
Any primary symptom	2.6	4.0	6.3	9.7	9.5	2.5	1.5	32.5	41.0	51.7	53.4	38.7
Rusty brown leaf fleck	0	0.8	0.6	0.4	18.7	23.8	18.7	0.6	0.2	2.7	18.3	37.8
Rusty brown stem fleck	0	0	0.4	0.8	7.6	9.3	4.6	0	0	0	7.1	26.6
Grey leaf spot	0	0	0	0	0	0	0	0	0	0	0	15.4
Leaf base lesion	0	0.4	0.4	0.4	0.6	3.6	3.1	0	0	0	13.2	21.3
Yellow leaf edge	0	0.8	1.0	0.5	9.5	10.9	18.3	0	0	0	12.0	39.0
Dead leaf	0	0	0.4	3.6	14.5	30.7	39.2	0	0	0	7.8	57.0
Grassiness	-	-	-	0.8	1.7	0.8	4.0	0	7.8	15.1	10.1	-
Leaf height (cm)	10	20	20-30	25-30				5	15-20	20-30	25-30	
Growth stage[d]	0	1	2/3	3/4	5			0	1	2/3	3/4	5

[a] Underground leaf sheath lesions were not scored as plants were left to grow for two seasons.

[b] Each cluster may be scored with more than one symptom.

[c] Not determined

[d] Key:

TABLE 2.7 The incidence of smoulder symptoms (per shoot) in a two year old experimental planting of narcissus, cv. Verger

Symptom [a]	% of shoots with symptom [b] (1979)				
	3/4	20/4	7/5	28/5	22/6
No shoot emergence	3.7	2.1	2.1	- [c]	-
Leaf tip distortion	1.5	3.0	6.8	4.5	-
Leaf tip lesion	8.0	8.1	10.2	12.2	10.9
Sickled leaf with lesion	0.6	0.9	1.5	0.7	-
Sickled leaf, no lesion	1.4	0.5	0.7	0.5	-
Collapsed shoot	1.0	0.1	0.2	0.3	0.6
Any primary symptom	12.6	12.2	17.9	18.4	10.9
Rusty brown leaf fleck	0	0	1.5	26.5	14.2
Rusty brown stem fleck	0	0	0	8.4	8.5
Grey leaf spot	0	0	0	0	4.0
Leaf base lesion	0	0	0	4.0	5.5
Yellow leaf edge	0	0	0	3.0	12.8
Dead leaf	0	0	0	1.7	19.5
Grassiness	-	-	11.6	7.7	-
Number of shoots	1684	2031	2112	2128	-
Number of leaves	5998	9329	10033	10172	-
Leaf height (cm)	5	15-20	20-30	25-30	-
Flower stage [d]	0	1	2/3	3/4	5

[a] Underground leaf sheath lesions were not scored as plants were left to grow for two seasons.

[b] Each shoot may be scored for more than one symptom.

[c] Not determined.

[d] See footnote in Table 2.6.

(Table 2.6). The corresponding figures in 1979 were strikingly higher at 19.0% and 37.7%. Similar but smaller increases were found during each season for the symptoms leaf tip distortion and sickled leaf lesion. The incidences of sickled leaves without a lesion and collapsed shoots both remained at low levels during 1978 (0-0.5%) and 1979 (0.4-4.2%). Approximately 1% of bulb clusters in 1978 and 2.1% in 1979 failed to produce shoots. The proportion of shoots showing any of the symptoms mentioned above - probable primaries - rose from 2.6% to 9.7% in 1978 (March to May) and from 32.5% to 51.7% (April to May) in 1979.

In both 1978 and 1979 the incidence of rusty-brown leaf flecking was low from March to May but rose rapidly in late May/June to c. 18% of clusters in 1978 and 40% in 1979. The incidence of rusty-brown stem flecks, leaf base lesions and shoots with one or two dead leaves also rose sharply after flowering. Flower spotting was noted on about 1% of flowers in 1978. The time of their occurrence again suggested that the above symptoms were caused by conidia of B. narcissicola released from sporulating mycelium in primary lesions.

Sclerotia were found infrequently in dying leaves and flower stalks towards the end of both seasons. Neither germinated sclerotia nor ascospores were observed in the field.

When the 1979 results were expressed as a proportion of the emerged shoots (Table 2.7), rather than of clusters, the incidences of all primary symptoms were lower. This

reflects the observation that usually only one or two shoots within an affected cluster emerge as smoulder primaries. In contrast, rusty-brown leaf flecking was found on a higher proportion of shoots (26.5% in June) than of clusters (18.3%) indicating that secondary infection was localised, most shoots within a cluster being affected. When expressed as a proportion of the emerged shoots, the incidence of leaf tip lesions, the most common primary symptom, remained at c. 10% throughout 1979. The rise in the number of clusters with a primary symptom up to early May would appear, from shoot and leaf counts, to result from the late emergence of infected shoots in previously healthy clusters.

(iii) Sclerotia in bulb scales. The incidence of bulbs bearing sclerotia was determined for several bulb lots. The results are presented in Table 2.8 with the bulbs classified by the symptom of the corresponding plant. Sclerotia were found significantly more frequently ($P < 0.05$) in the bulbs of plants considered to be primaries (23%) or in the bulbs of plants with symptoms of secondary infection (14%) than in bulbs without shoot symptoms (9%). Sclerotia were also significantly more frequent ($P < 0.05$) in the bulbs of plants with primary symptoms than secondary symptoms.

D. Conclusions

From the above observations and isolations, lesions at the leaf tip and in sickle-shaped leaves may be regarded as typical primary symptoms, particularly when found on more than one leaf in a shoot, at similar heights above the ground.

TABLE 2.8 The occurrence of botrytis sclerotia in the bulbs of plants with foliage smoulder

Sampling date	Source of bulbs	Cultivar	Shoot symptom	Time in ground (yrs)	Sample size	% bulbs bearing sclerotia
October 1978	GG[a]	Sempre Avanti	-	0	1000	6.3
October 1978	GG	Golden Harvest	-	0	400	4.0
March 1979	Cushnie	Golden Harvest	None	3	50	2.0
			Primary	3	100	11.0
June 1979	Cushnie	Golden Harvest	None	3	50	0
			Primary	3	25	64.0
			Leaf fleck lesion	3	25	8.0
			Leaf base lesion	3	25	16.0
July 1979	Cushnie	Golden Harvest	None	3	25	12.0
			Leaf fleck lesion	3	25	8.0
			Leaf base lesion	3	25	16.0
			Rotting stem	3	25	12.0
September 1979	SHRI	Verger	None	2	98	17.0
			Primary	2	154	24.0
			Leaf fleck lesion	2	87	17.0
January 1980	ESCA	Carlton	Primary	3	20	20.0
Combined results [b]			Before planting		1400	5.6
			No symptoms in the shoot		223	9.0a
			Smoulder primary symptom		299	22.7b
			Smoulder secondary symptom		212	14.0 a,c

[a] Grampian Growers Ltd., Montrose.

[b] Percentages followed by different letters are significantly different ($P < 0.05$).

Failure to emerge, complete rot at emergence (collapse) and flower bud lesions are also primary symptoms, though less frequently encountered. Leaf distortion, sickled leaves without a lesion and grassy shoots may all result from B. narcissicola infection but also have well documented alternative causes. Symptoms of secondary infection by B. narcissicola are (1) dark brown rots in flower stalk ends and broken leaves, (2) rusty brown flecks, dark brown spots and streaks on leaves and (3) leaf base lesions, occurring late in the season and usually in only one leaf within the shoot. Isolated leaf tip lesions and flower spotting occurring late in the season result from infection by B. narcissicola or B. cinerea, whereas grey leaf spots, although sometimes containing Botrytis spp., are probably caused by some other agent. Symptoms of secondary infection increase after flowering and as leaves begin to senesce.

Sclerotia are found most frequently in the bulbs of plants with primary symptoms of smoulder. However, botrytis sclerotia in the outer bulb scale cannot be taken as indicative of B. narcissicola infection in the bulb because some are probably B. cinerea and others are not viable.

2. The origin of primary symptoms

A. Infected bulbs

One hundred plants (cv. Golden Harvest) with primary symptoms and 50 apparently healthy plants were lifted from a three year old planting at Cushnie Farm in March 1979 and the bulbs examined for sclerotia and sampled for B. narcissicola by isolation (Table 2.9). Botrytis narcissicola

TABLE 2.9 The occurrence of B. narcissicola in the bulbs of plants with primary symptoms of smoulder

Shoot[a] symptom	Number in sample	Sclerotia in[b] outer scales (%)	Isolation of B. narcissicola (%) from Outer scales	Fleshy scale	Bulb[c] neck	Anywhere
Healthy	50	2	10	0	13	23
Primary[d]	100	11 NS	17	9	40	40 *

[a] Plants were dug up on 28/3/79.

[b] Sclerotia and all isolates were grown on PDA for identification; none were B. cinerea.

[c] Tissues sampled at the bulb neck were the bases of leaf sheaths, leaves and flower stalks.

* significantly different from the control ($P < 0.05$).

NS not significant.

was isolated significantly more frequently ($P < 0.05$) from bulbs with infected shoots than bulbs with healthy shoots, the pathogen being located mainly in the leaf, flower stalk and sheath bases of the bulb neck.

Ten weeks later further samples of plants with infected or healthy shoots were dug up. Sclerotia were found in the bulbs of most plants with infected shoots but in none of the bulbs of apparently healthy plants. The sclerotia were located in the bulb neck (72% of bulbs) and the outer scales (64% of bulbs). The increase in the incidence of sclerotia in the bulbs of plants with primary symptoms, from 11% to 72%, suggests that infection within the bulb may have progressed during the growing season.

The finding that shoots with primary symptoms often have bulbs infected with B. narcissicola supports the hypothesis that primaries develop from infected bulbs. The development of shoots from bulbs inoculated naturally or artificially with B. narcissicola was therefore investigated.

On the assumption that numerous sclerotia in the outer scales is a symptom of infection, 48 infected bulbs (cv. Sempre Avanti, 12-15cm) were planted in October 1978. Bulbs from the same stock but without evident sclerotia were planted as a control. The following spring only 6% of bulbs bearing sclerotia produced shoots with symptoms of smoulder, the same frequency as bulbs without sclerotia.

In further experiments bulbs were artificially inoculated with B. narcissicola to ensure infection. Small (3-4cm) VT bulbs, cv. Sempre Avanti, were inoculated at the neck with

mycelium before planting (11/12/78). By June of the following year all 24 control bulbs, inoculated with agar alone, had produced leaves 10-15cm in length, but none of the bulbs inoculated with B. narcissicola had emerged.

Large (12-15cm) bulbs, cv. Golden Harvest, were wound-inoculated with B. narcissicola mycelium, either in the neck or just above the basal plate, and planted (October 1978) when a lesion had developed in the fleshy tissue. All bulbs, except one inoculated at the neck with B. narcissicola, produced shoots. By mid-May 29% of bulbs would-inoculated at the neck and 21% of bulbs wound-inoculated at the base had produced some shoots with primary smoulder symptoms, mostly leaf tip lesions (Table 2.10). With the inclusion of poor shoot growth as a primary symptom the figure for neck-inoculated bulbs rose to 35%. For bulbs inoculated with mycelium, at both the neck and base, the frequency of primary smoulder symptoms was significantly greater ($P < 0.001$ and $P < 0.01$ respectively) than in the shoots of corresponding agar-inoculated bulbs. Inoculation at the bulb neck caused more primary symptoms than inoculation at the base ($P < 0.05$).

B. Infested soil

Small (3-4cm) VT bulbs, cv. Sempre Avanti, were hand-planted in October 1978 with 0.75g of outer bulb scales, bearing a minimum of 10 botrytis sclerotia, positioned in the soil 2cm above bulb noses. The following spring (25/5/79), 27% of the bulbs had produced shoots with smoulder symptoms: 8% with leaf tip lesions, 4% with sickled leaves, 2% with dead leaves and 13% not emerged. The corresponding figures for a control treatment, without debris, were 4% leaf tip lesions and 2% not emerged, the difference in the frequency of total

TABLE 2.10 The development of shoots with primary smoulder symptoms from bulbs inoculated with B. narcissicola

Recording date	Bulb Inoculation	Number of plants (of 48) with symptoms[a]						% plants with any symptoms
		Sickled leaf with lesion	Sickled leaf, no lesion	Leaf tip lesion	Collapsed shoot	Leaf distortion	Small leaves	
2/5/79	Agar in neck	0	1	3	0	0	[b]	8
	Mycelium in neck	1	1	8	1	1	-	23
	Agar in base	0	1	3	0	0	-	8
	Mycelium in base	2	2	7	0	0	-	21
25/5/79	Agar in neck	0	0	3	1	0	2	8
	Mycelium in neck	1	0	9	5	0	6	35***
	Agar in base	0	1	1	1	0	2	10
	Mycelium in base	0	1	7	0	0	6	21**

[a] 100% emergence in all treatments except for mycelial inoculation at the bulb neck (98%).

[b] Not recorded.

** Significantly different ($P < 0.01$) from agar inoculated bulbs.

*** Significantly different ($P < 0.001$) from agar inoculated bulbs.

symptoms (27% against 6%) being highly significant ($P < 0.001$).

In a second experiment small VT bulbs (3-4cm; cv. Sempre Avanti) were planted 10cm deep in 15cm pots, with groups of five B. narcissicola sclerotia positioned either in contact with a bulb nose or in peat 2cm directly above a bulb nose. The pots were plunged to the rim in Stirling University gardens. The following spring (12/4/80) sclerotia had not significantly affected ($P > 0.05$) shoot emergence. Of 33 bulbs in each treatment, 45% with sclerotia positioned on the nose and 36% with sclerotia above the nose failed to emerge; all emerged shoots lacked obvious smoulder symptoms. When bulbs were lifted (7/7/80) sclerotia were observed in scales at the neck, in 18 and 24% of those inoculated on and above the nose respectively, indicating successful infection of bulbs from B. narcissicola sclerotia.

C. Associated leaf sheath lesions

Plants with primary symptoms (112) or apparently healthy shoots (38) were lifted from a third year planting of Golden Harvest in March 1979 and examined. The frequencies of lesions in leaf sheaths and in bulb necks associated with infected and healthy shoots are given in Fig. 2.1. Leaf sheath lesions were found most commonly in plants with primary symptoms; only 13% of shoots with primary symptoms lacked a sheath lesion (Plate 2.4).

From these experiments, infected bulbs would appear to be the most likely source of shoots with primary symptoms. The development of a lesion in the sheath seems to be an important stage in the progress of infection from bulb to

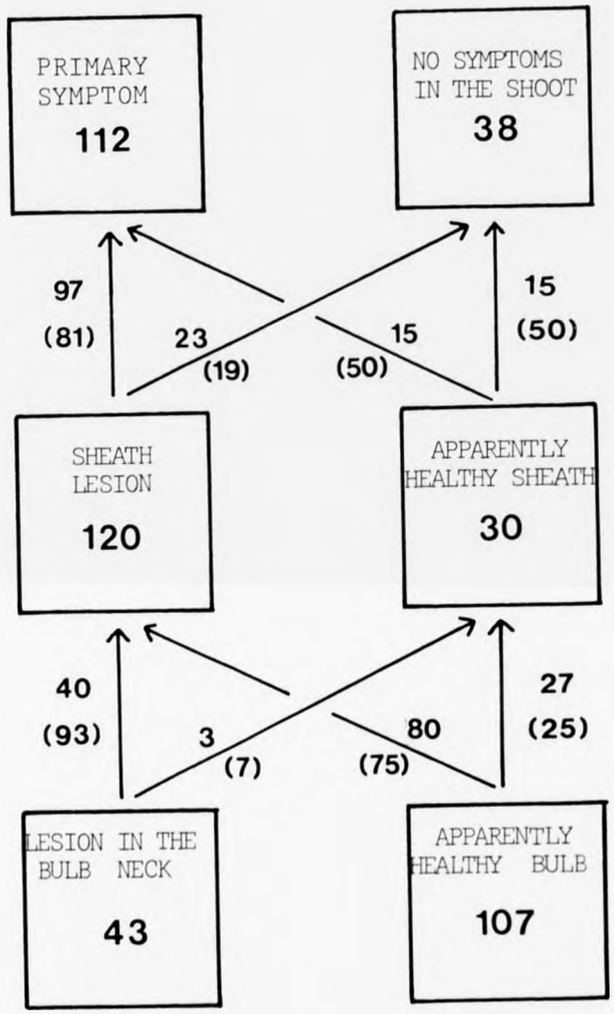


FIG. 2.1 The association of lesions in the leaf sheath and bulb neck with primary symptoms of smoulder. Plants with primary symptoms (112) and apparently healthy plants (35) were lifted (28/3/79) and examined. Figures in boxes show the total number of plants with each symptom. Figures alongside arrows, probable routes of infection development, indicate the number of plants with symptoms of the two connected boxes; figures in parentheses are percentages. Note that the majority of plants with primary symptoms (97 of 112) also have sheath lesions.

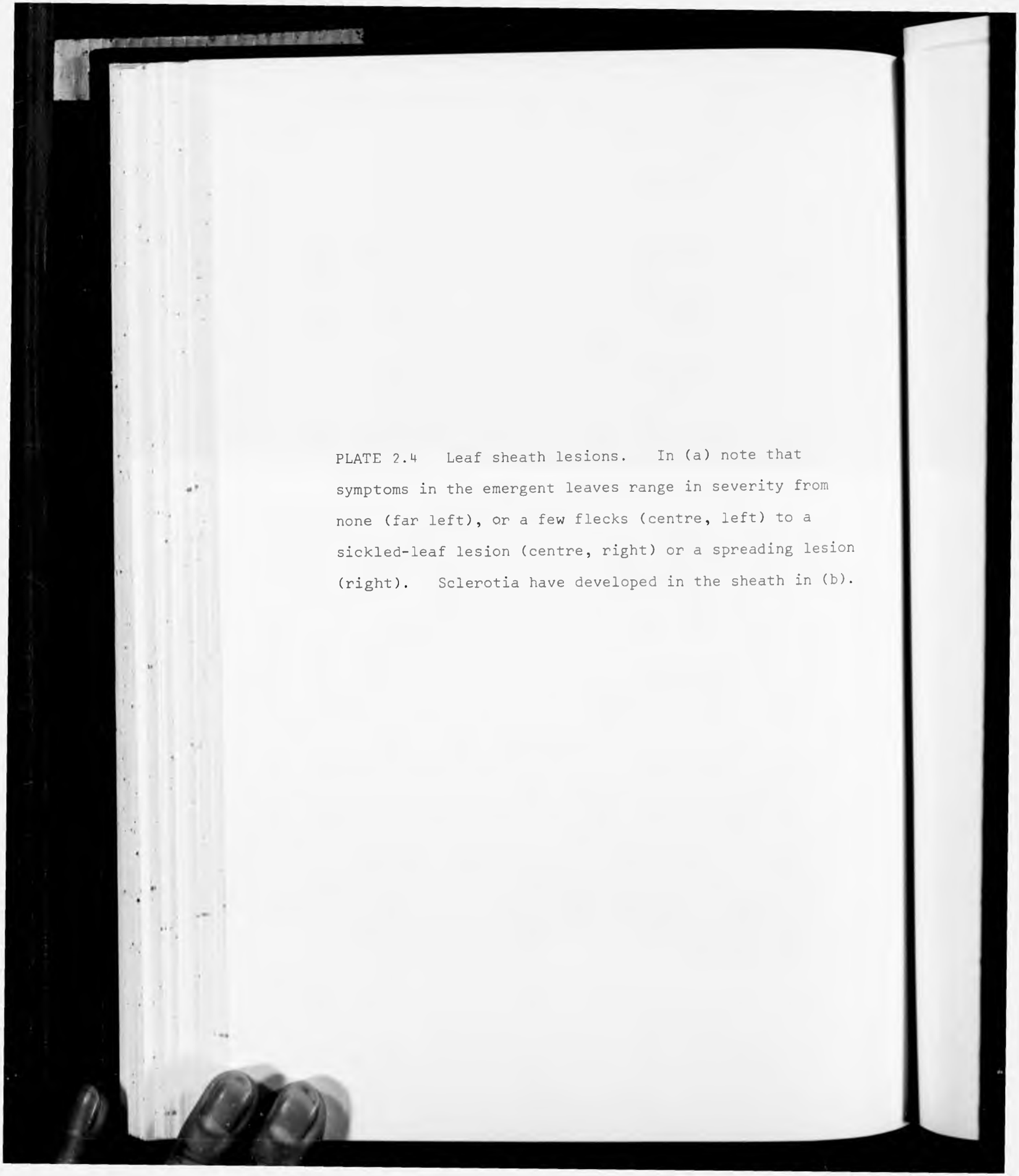


PLATE 2.4 Leaf sheath lesions. In (a) note that symptoms in the emergent leaves range in severity from none (far left), or a few flecks (centre, left) to a sickled-leaf lesion (centre, right) or a spreading lesion (right). Sclerotia have developed in the sheath in (b).

In (a) note that
ge in severity from
entre, left) to a
or a spreading lesion
in the sheath in (b).

a.

b.

PLATE 2.4

In (a) note that
ge in severity from
entre, left) to a
or a spreading lesion
in the sheath in (b).

a.

b.

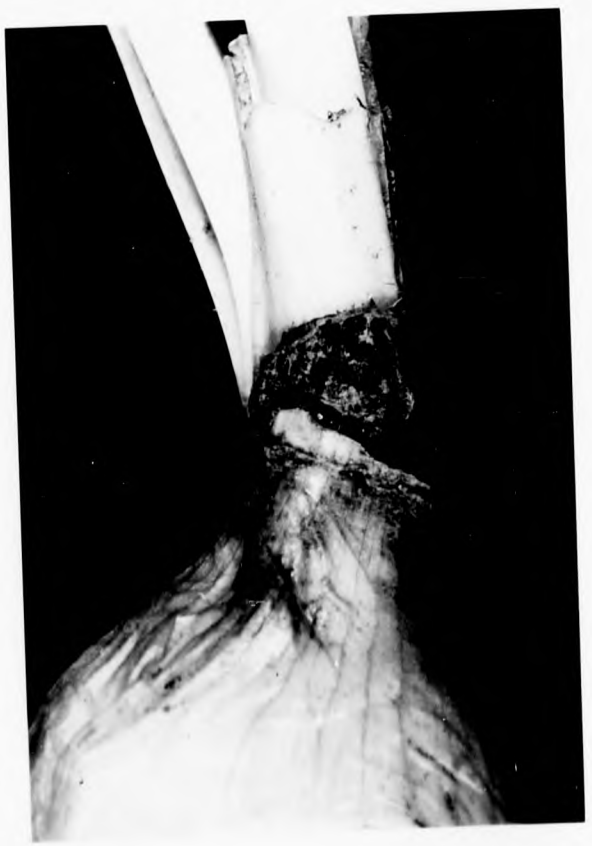


PLATE 2.4

shoot. Botrytis narcissicola sclerotia and infected debris in the soil can also cause primary symptoms and may be important inocula where bulbs are left in the ground, undisturbed, for many years.

3. Secondary infection of shoots

In an attempt to define the different conditions which may lead to secondary infection of narcissus by B. narcissicola, a series of infection experiments was carried out on plants growing in the field. Leaves and flower stalks were inoculated with conidia and/or mycelium. Both healthy and wounded tissues were inoculated and the influence of adding nutrients (V8 juice) to conidial inocula was examined.

A. Infection of leaves from conidia of B. narcissicola

Leaves of narcissus, cv. Sempre Avanti, were spray-inoculated on 29/5/79. One week after inoculation the plants remained without symptoms. After 2 wks dark brown lesions, 0.1-1cm in length, were present at wound sites on all plants inoculated with B. narcissicola whereas no lesions developed at wound sites inoculated with SDW or V8 juice alone. Three weeks after inoculation wound site lesions were classified as either limited (< 2mm spread) or spreading; the majority of plants wound-inoculated with B. narcissicola possessed spreading lesions and leaves were withering rapidly (Table 2.11).

Fleck lesions, leaf tip lesions and leaf base lesions were apparent in the leaves of several plants in all treatments 3 wks after inoculation (Table 2.11). On unwounded leaves, tip lesions were more frequent in

TABLE 2.11 Leaf infection from B. narcissicola conidia [a]

Inoculation [b]			Number of plants (of 48) developing symptoms [c]						
Wounding	Nutrients	Conidia	Fleck lesion	Tip lesion	Base lesion	Dead leaf	Wound site lesion: Limited	Wound site lesion: Spreading	% plants with any symptom
Unwounded	Water	-	8	8	0	1	NA [d]	NA	35
		+	8	17	1	2	NA	NA	58**
	V8-juice	-	9	10	0	1	NA	NA	42
		+	13	16	1	4	NA	NA	71***
Wounded	Water	-	0	13	0	0	2	1	27
		+	- [e]	0	0	0	7	41	100***
	V8-juice	-	10	8	0	0	2	0	38
		+	-	0	0	4	1	46	100***

[a] See also Plates 2.5 and 2.6.

[b] See text for details.

[c] Symptoms recorded three weeks after inoculation (19/6/79); more than one symptom recorded on some plants.

[d] Not applicable.

[e] Flecking not recorded because of rapid leaf withering, due to infection at the wound site.

** Significantly different from control ($P < 0.01$).

* ** Significantly different from control ($P < 0.001$).

B. narcissicola inoculated plants (17 of 48) than plants of the corresponding control treatment (9 of 48). Fleck lesions occurred to a similar extent in treatments with or without conidia. Lesions at the leaf base were occasionally seen in B. narcissicola inoculated plants. The number of unwounded plants with any lesion was significantly greater ($P < 0.01$ and $P < 0.001$) for plants inoculated with B. narcissicola conidia than for control plants inoculated with SDW or V8 juice. There was no significant difference ($P > 0.05$) in lesion formation resulting from inoculation with conidia in SDW compared with V8 juice, in either wounded or unwounded plants.

Five weeks after inoculation leaf death in those blocks of plants inoculated with B. narcissicola conidia was noticeably greater than in corresponding control treatments (Plates 2.5 and 2.6).

B. Infection of leaves from mycelium of B. narcissicola Leaves of narcissus, cv. Golden Harvest, were inoculated on 24/4/79 with B. narcissicola mycelium, either directly or after wounding with a needle. No lesions were evident on removal of polythene incubation bags (24h). One week after inoculation 86% of B. narcissicola inocula on wounded leaves, compared with 2% on unwounded leaves, had caused spreading lesions (Table 2.12). Lesions extended further in the direction of the leaf tip (5-10cm) than the leaf base (1-2cm), an effect not noticed following inoculation with conidia. A few spreading lesions (5 of 48) developed at control wound sites in this experiment. Four weeks after inoculation all leaves wound-inoculated with B. narcissicola were dead from the wound site upwards; no

a.

b.

PLATE 2.5 Premature leaf senescence in plants (unwounded) inoculated 5 wks previously with conidia of B. narcissicola in V8 juice (a). Note the leaf flecking and dead leaves. Plants in (b) were inoculated with V8 juice alone.

a.

b.

PLATE 2.5 Premature leaf senescence in plants (unwounded) inoculated 5 wks previously with conidia of B. narcissicola in V8 juice (a). Note the leaf flecking and dead leaves. Plants in (b) were inoculated with V8 juice alone.

a.

b.

PLATE 2.6 Premature leaf senescence in plants inoculated 5 wks previously with conidia of B. narcissicola in SDW into wounded leaves (a). Note the numerous dead leaves in (a). Plants in (b) were inoculated with water alone.

a.

b.

PLATE 2.6 Premature leaf senescence in plants inoculated 5 wks previously with conidia of B. narcissicola in SDW into wounded leaves (a). Note the numerous dead leaves in (a). Plants in (b) were inoculated with water alone.

TABLE 2.12 Leaf infection from B. narcissicola mycelium

Inoculation[a]		Plants developing spreading lesions[b]		Lesions extending more to tip than base[b]		Leaf rot to soil level[c]	
		Number	%	Number	%	Number	%
Unwounded	Agar	0	0	0	0	2	4
	Mycelium	1	2	1	2	2	4
Wounded	Agar	4	10	1	2	1	3
	Mycelium	35	86	30	75	8	20

[a] See text for details.

[b] Recorded on 1/5/79, one week after inoculation.

[c] Recorded on 19/6/79, eight weeks after inoculation.

[d] Results adjusted to allow for a small number of inocula not remaining in contact with the leaf for at least 24h.

lesions had by then reached soil level. After a further 4 wks 20% of wound-inoculated leaves were dead down to the soil surface (Table 2.12).

Because of the conflicting results obtained for lesion development following the inoculation of detached and attached leaves (cf. Tables 1.3 and 2.12) the influence of leaf preparation on the susceptibility of detached leaves was investigated. Detached leaves, cv. Golden Harvest, were inoculated (1), directly after collection (2), following a light rinse in tap water and (3) following repeated rubbing with a soft, damp tissue, with conidia or mycelium of B. narcissicola. There was no significant difference between treatments in the number of spreading lesions recorded 5 days after inoculation. The majority of conidial inocula caused limited lesions while c. 50% of mycelial inocula, in all treatments, caused spreading lesions.

C. Infection of flower stalks from conidia of B. narcissicola and B. cinerea

Cut flower stalks, cv. Sempre Avanti, were inoculated with conidial suspensions of B. narcissicola and B. cinerea in SDW on 1/5/79. No symptoms were visible 1 wk after inoculation but after 3 wks all B. narcissicola inoculations had caused a dark brown rot extending at least 2cm down the stalk and 29% of stalks had rotted down to soil level. The corresponding figures for B. cinerea inoculated stalks were 69% with lesions >2cm and 4% to soil level (Table 2.13). Only 15% of stalks inoculated with SDW (control) had developed lesions >2cm in length. Botrytis sporulation was most frequently seen on stalks inoculated with B. narcissicola and very rarely in B. cinerea or

TABLE 2.13 Rotting of flower stalks following inoculation with B. narcissicola or B. cinerea conidia

Time after inoculation (wks)	Inoculum[a]	Extent of flower stalk rot % inoculations in each category				% plants with (i) sporulation on flower stalk	(ii) lesion in adjacent leaf
		No rot	<2cm	>2cm	To soil		
3	Water	35	50	10	5	6	4
	B. cinerea	8	19	69	4	1	15*
	B. narcissicola	0	0	71	29	96	42***
5	Water	8	33	44	15	17	4
	B. cinerea	6	4	56	34	25	8
	B. narcissicola	0	4	46	50	81	33***
7	Water	[b]	-	-	46	-	23
	B. cinerea	-	-	-	60	-	15
	B. narcissicola	-	-	-	83	-	46**

[a] One flower stalk per plant inoculated with 0.5ml of a suspension of 5×10^5 spores per ml in SDW.

[b] Not recorded.

* Significantly different from control ($P < 0.05$).

** Significantly different from control ($P < 0.01$).

*** Significantly different from control ($P < 0.001$).

water-inoculated stalks (Table 2.13).

In subsequent examinations the extent of stalk rotting in all treatments had increased (Table 2.13). Seven weeks after inoculation 83% of B. narcissicola, 60% of B. cinerea and 46% of water-inoculated stalks had rotted to soil level. Botrytis sporulation was now more evident on B. cinerea-inoculated stalks and was also present in rotting control stalks. As stalk rots progressed downwards lesions frequently developed in leaf bases adjacent to the stalk (Plate 2.3a). Such leaf lesions were most common following B. narcissicola stalk inoculation and were significantly greater ($P < 0.01$) than in plants inoculated with B. cinerea or water alone (Table 2.13).

4. Infection of bulbs

A. Isolation of B. narcissicola from the bulbs of plants with symptoms of secondary infection

The spread of secondary infections down leaves and flower stalks provide two possible routes by which previously healthy bulbs may become infected. To test this hypothesis, plants which were recorded as healthy at the beginning of the season and which later developed secondary infection symptoms were sampled for B. narcissicola in various parts of the bulb.

In a three year old planting of cv. Golden Harvest at Cushnie Farm, a plot (7 adjacent ridges, each 20m long) was regularly inspected and plants with primary symptoms were marked by stakes. The bulbs of other plants, which developed secondary symptoms or remained healthy were

hand-lifted in July (following foliage die-down) after carefully tracing shoots back to their correct nose of origin within bulb clusters. The bulbs were dissected and small pieces of tissue - the bases of leaf sheaths, leaves and flower stalks, and the tip of next season's flower bud - were sampled for B. narcissicola by isolation (Fig. 2.2).

Botrytis narcissicola was isolated significantly more frequently ($P < 0.01$) from the bulbs of plants which had shown secondary infection symptoms than from the bulbs of apparently healthy plants (Table 2.14). For example, B. narcissicola was isolated from the bases of 72% of flower stalks which had rotted after picking, compared with 12% from the bases of stalks not picked at flowering. Botrytis narcissicola was also isolated from the abscission zone at the bases of the dead leaf sheaths and leaves within the bulb neck. The fungus was not isolated from the new season's flower bud and was isolated infrequently from fleshy bulb tissue a few millimetres below abscission zones.

In a similar experiment carried out before complete foliage death, B. narcissicola was found in 92% of the bulbs of plants with a leaf base lesion, compared with 40% of the bulbs from apparently healthy plants or plants with fleck lesions (Table 2.14).

B. Isolation of B. narcissicola from VT bulbs

In 1979 smoulder was found in some plants of three year old VT bulbs in gauzehouses at SHRI and ESCA. Sclerotia were frequently present in leaf sheaths and B. narcissicola was usually isolated from the bulb necks. Clones of VT bulbs,

hand-lifted in July (following foliage die-down) after carefully tracing shoots back to their correct nose of origin within bulb clusters. The bulbs were dissected and small pieces of tissue - the bases of leaf sheaths, leaves and flower stalks, and the tip of next season's flower bud - were sampled for B. narcissicola by isolation (Fig. 2.2).

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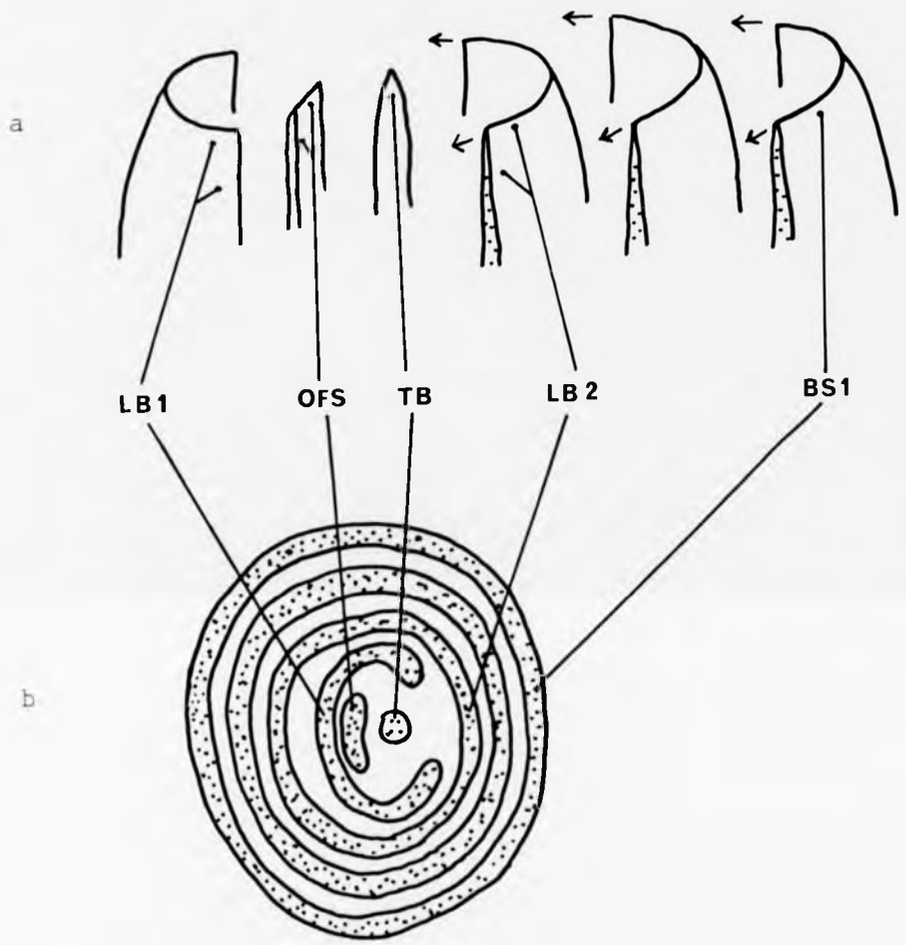

FIG. 2.2 Diagram showing tissues sampled for B. narcissicola by isolation. View (a) and transverse section (b) of a bulb neck (drawn expanded) showing the relative positions of the terminal bud (TB), old flower stalk (OFS), leaf bases (LB) and bulb scales (BS).

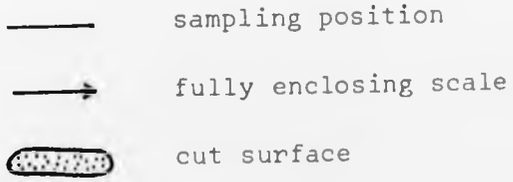

TABLE 2.14 Isolation of B. narcissicola from the bulbs of plants with secondary infection symptoms

Symptom	Sample size	% samples yielding B. narcissicola					
		Bulb neck			Terminal bud	Outer scale	Anywhere in the bulb
		Sheath base	Leaf base	Stalk base			
Experiment 1 [a]							
Apparently healthy	25	16	28(4)[c]	12(0)	0	-[d]	48
Leaf fleck lesion	25	32	44(4)	32(0)	0	-	80**
Leaf base lesion	25	44	56(20)	44(16)	0	-	84**
Rotting flower stalk	25	36	40(4)	72(0)	0	-	88**
Experiment 2[b]							
Apparently healthy	45	24	11	-	-	4	40
Leaf fleck lesion	25	24	24	-	-	20	40
Leaf base lesion	25	72	44	-	-	24	92***

[a] Complete foliage dieback allowed before bulbs of the marked plants were lifted (12/7/79).

[b] Bulbs lifted before complete dieback (6/6/79).

[c] % isolation success from fleshy tissue about 5mm below abscission zones are given in parenthesis.

[d] Not recorded.

** Differs significantly from the control ($P < 0.01$).

*** Differs significantly from the control ($P < 0.001$).

obtained from a benomyl-treated mother bulb by twin-scaling and grown in sterile soil, ought, at least initially, to be free from B. narcissicola. Therefore, infection of VT bulbs probably developed following colonisation of leaves or flower stalks by B. narcissicola.

The results of the above experiments support the hypothesis that the secondary infection of shoots leads to the infection of previously healthy bulbs with B. narcissicola.

5. Seasonal carryover

A. Survival of B. narcissicola mycelium in bulbs

An ability to survive in the bulb, in a form other than the readily detected sclerotia in outer scales or in a progressive rot, may explain the occurrence of plants with sheath lesions and primary symptoms of smoulder in the shoot but apparently healthy bulbs (Section 2.2C). Results from Section 2.4A suggest that B. narcissicola occurs in the bulb neck, the fungus primarily remaining as a saprophyte in dead leaf and flower stalk bases. As dry storage of bulbs after lifting would seem to be a time at which the level of such an inoculum may fall, a comparison was made on the survival of B. narcissicola in bulbs stored dry or moist.

Restricted lesions were established in small (4-6cm) VT bulbs, cv. Sempre Avanti, by inoculating wounds with B. narcissicola mycelium and storing bulbs in moist or dry conditions. After 6 wks the majority of lesions in both wet and dry-stored bulbs had increased in size from 1 to c. 1.5cm diameter, and in both treatments 16% of bulbs had

completely rotted. Botrytis narcissicola was recovered less frequently from dry-stored bulbs (18 of 25) than wet-stored bulbs (23 of 25), but this difference was not significant ($P > 0.05$).

It would appear that B. narcissicola mycelium can survive in bulbs for at least 6 wks during dry storage.

B. Survival of B. narcissicola sclerotia buried in soil

Bags of sclerotia, mixed with a small amount of sand, were buried in a loam soil at SHRI in December 1978 and September 1979. At 6 or 12 wk intervals three bags were removed (each experiment) and the recovered sclerotia assessed for viability with or without a surface sterilisation treatment (Table 2.15 and Fig. 2.3).

The two methods of assessing viability gave similar results. The number of sclerotia producing mycelium was slightly lower following surface-sterilisation than following rinses in SDW (Table 2.15) but this difference was not significant (F statistic, D.F. 1,28; $P > 0.05$). The means of all viability tests were therefore used in calculating survival i.e. mean persistence x mean viability. Fungal contaminants developed occasionally, particularly from sclerotia not surface sterilised, but they were generally overgrown by the mycelium of B. narcissicola on V8 juice agar discs.

In the first experiment survival of B. narcissicola sclerotia fell significantly, to c. 75% after burial for 26 wks, and in the second experiment to a similar level within 12 wks (Fig. 2.3). After 9 months less than 40%

TABLE 2.15 Effect of burial in soil on the persistence and survival of B. narcissicola sclerotia

Experiment	Sampling date	Number of weeks buried	% germinated[a] in soil	Persistence[b] (%)	Viability[c] (%) A	Viability[c] (%) B	Survival[d] (%)
1	31/1/79	7	0	97	100	90	93a
	15/3/79	13	0	100	100	97	98a
	17/4/79	18	0	100	100	93	97a
	12/6/79	26	10	83	100	82	75b
	27/7/79	32	12	97	86	60	72b
	13/9/79	39	12	72	67	41	38c
	14/12/79	52	-	65	68	60	42c
	5/3/80	63	-	50	-	50	25c
2	5/1/80	12	0	83	92	86	74a'
	5/3/80	24	0	88	74	61	60b'
	10/6/80	36	0	53	76	64	37c'

[a] Producing conidiophores.

[b] Mean number of sclerotia recovered from three replicate bags.

[c] Mean germination of recovered sclerotia, recorded 5 days after plating onto V8 juice agar, without (A) or with (B) surface sterilisation.

[d] Mean number of viable sclerotia (persistence x $\frac{\text{viability A} + \text{viability B}}{2}$)

Figures followed by different letters are significantly different (Duncan's new multiple range test, $P < 0.05$).

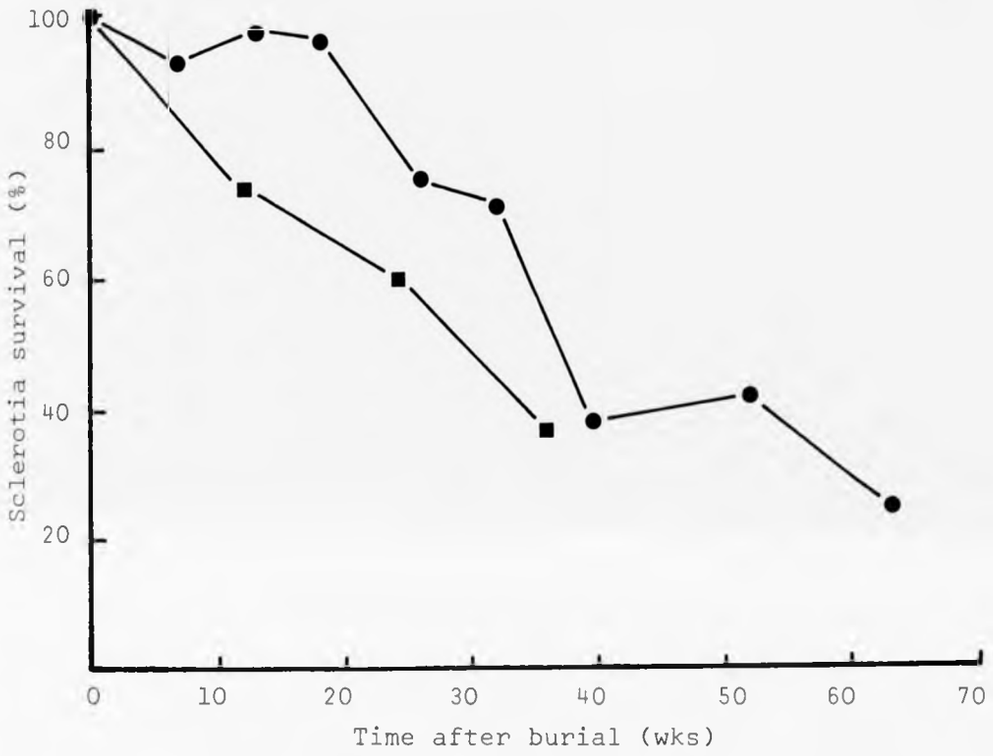

FIG. 2.3 Decline in the survival of B. narcissicola sclerotia buried in soil. Sclerotia were mixed with sand, enclosed in nylon bags and buried 10cm deep. Three replicate bags were dug up at intervals and sclerotial persistence and viability were determined (see text for details). Survival was calculated from the formula: mean persistence x mean viability of recovered sclerotia.

●——● Experiment 1; sclerotia buried December 1978

■——■ Experiment 2; sclerotia buried September 1979

of sclerotia recovered were viable. Some sclerotia were observed to have germinated, producing conidiophores, after 26 wks burial (Table 2.15).

C. Relationship between primary and secondary symptoms in plants observed during two seasons

In April 1978 all clusters (c. 500) in the infected planting of cv. Verger at SHRI were examined for the presence or abscence of shoots with primary symptoms of smoulder. Clusters not marked as primaries were examined again in June and classified as either healthy or showing symptoms of secondary infection. In the second season, clusters with primary symptoms were recorded in April.

The incidences of primary and secondary symptoms are recorded in Fig. 2.4. The frequency of primaries increased from 6% of clusters in 1978 to 33% in 1979. The majority of first season primaries (62%) emerged as primaries in the second season, and the majority of second season primaries (66%) were recorded as infected, either primary or secondary symptoms, in the first season. However, only 35% of all the plants recorded with symptoms of secondary infection in 1978 emerged infected in 1979, a figure only slightly greater (significant at $P = 0.05$) than the number of apparently healthy plants in 1978 emerging with primary symptoms in 1979 (27%).

Plants of cvs. Golden Harvest and Sempre Avanti, artificially inoculated in spring 1979 with conidia, mycelium or sclerotia of B. narcissicola, on the leaf, flower stalk or bulb (Sections 2.2 and 2.3), were examined for symptoms of primary infection in spring 1980. The occurrence of sheath

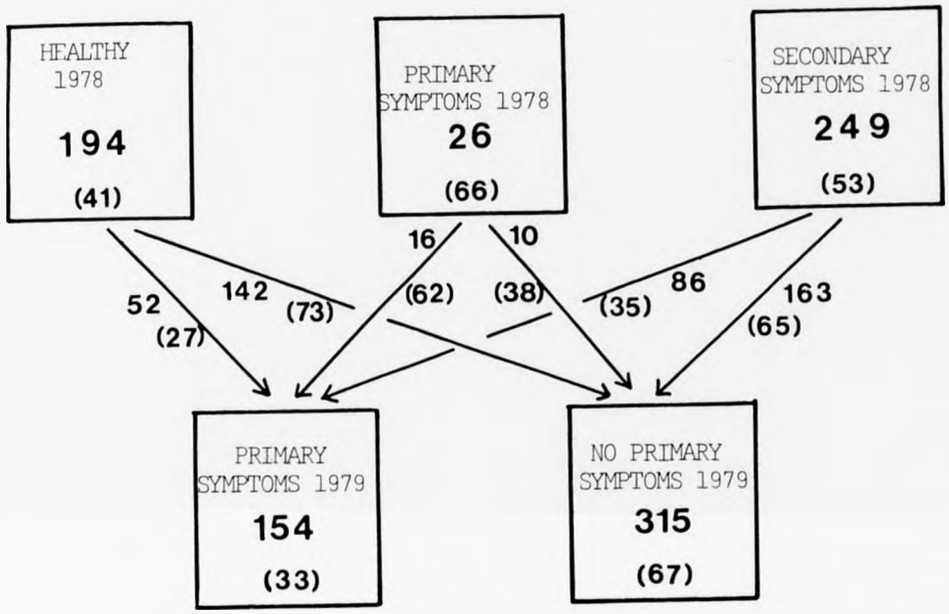

FIG. 2.4 Seasonal carryover of smoulder in an experimental planting of narcissus, cv. Verger. Boxes show the proportion of bulb clusters in different symptom categories recorded in 1978 and 1979. Figures in bold type are absolute numbers and figures in parentheses are percentages. Connecting arrows show the proportion of plants in each 1978 category recorded with and without primary symptoms in 1979. Note that many plants infected during 1978 (86 of 249) emerged with primary symptoms in 1979.

lesions was also recorded after lifting on 11/4/80 (Table 2.16).

The number of plants with primary symptoms was generally low but, including sheath lesions as a primary symptom, six treatments resulted in a significantly greater number of second season primary symptoms than their respective controls. Apart from conidia sprayed onto leaves in V8 juice, and sclerotial infection of bulbs, these were all wound-inoculation treatments which had caused spreading lesions in the first season.

The number of plants with sheath lesions was greater than the number of plants with leaf lesions, suggesting that transfer of infection from sheath to leaf or flower bud may be an important stage in determining whether or not shoots emerge with primary symptoms.

6. Factors influencing the incidence of plants with primary symptoms

Assuming that most plants with primary symptoms arise from infected bulbs, an investigation of some of the factors which may influence their development could help to explain the unpredictability of their occurrence. Three factors, depth and time of bulb planting and the type of growth medium, were examined.

A. Depth and time of bulb planting

Verger bulbs, selected as plants with a history of smoulder, were planted in a bed at SHRI at depths of 5, 15 and 25cm in September and at 15cm in December 1979. The plants were scored in April 1980 for (1), shoot emergence (2), primary

TABLE 2.16 Seasonal carryover of smoulder in plants inoculated with *B. narcissicola*

Inoculation in 1979			Number of plants (of 48) with primary symptoms in 1980[a]			
			Sheath lesion only	Leaf lesion only	Sheath and leaf lesion	Any lesion
1. Conidia on leaves						
conidia	wounded	V8 juice				
-	-	-	8	6	13	26
+	-	-	6	3	12	21
-	-	+	3	5	6	14
+	-	+	10*	2	4	16*
-	+	-	3	3	7	13
+	+	-	9*	15**	10	34**
-	+	+	0	6	10	16
+	+	+	10**	8	7	25*
2. Mycelium on leaves						
mycelium	wounded					
-	-		7	2	7	14
+	-		5	1	7	13
-	+		7	1	7	15
+	+		9	2	12	23*
3. Conidia in flower stalks						
Water			6	7	4	17
B. cinerea			9	2	4	15
B. narcissicola			7	10	8	25*
4. Mycelium in bulbs						
agar at neck			1	1	4	6
mycelium at neck			3	1	3	7
agar at base			2	0	1	3
mycelium at base			3	1	4	8
5. Sclerotia on bulbs (natural infection)						
no sclerotia evident			6	1	5	12
sclerotia			11	3	10	24**
6. Sclerotia in debris over bulb						
no debris			1	4	0	5
sclerotial debris			2	5	3	10

[a] Bulbs lifted and plants examined for symptoms in April, 1980.

* Significantly different from control ($P < 0.05$).

** Significantly different from control ($P < 0.001$).

symptoms and (3) after lifting, for leaf and sheath lesions below ground level and also bulb neck rot. Results obtained are given in Table 2.17. The only treatment which significantly influenced the frequency of lesion occurrence was shallow planting in September, causing a significant increase ($P < 0.001$) over the other three treatments.

B. The growing medium

Infected bulbs (12-14cm., cv. Verger) were planted in peat, loam or coarse sand in pots plunged in soil at Stirling University gardens in September 1979. When examined the following spring, plants with primary symptoms occurred more frequently in pots of loam (9 of 40) than sand (7 of 40) or peat (5 of 40) but differences were not significant ($P>0.05$).

TABLE 2.17 Influence of depth and date of planting on the development of shoots with primary smoulder symptoms from infected bulbs

Bulb size (cm)	Date planted	Depth (cm) planted	Number of plants with symptoms in 1980[a] Emerged[b] healthy	Not emerged	Neck rot	Sheath lesion[c] only	Leaf lesion[c] only	Sheath and[c] leaf lesion	Any[d] lesion
12-14	September	5	1	0	0	16	0	13	29
	September	15	4	4	0	19	0	3	22
	September	25	6	10	2	14	0	3	19
	December	15	1	24	2	4	3	5	14
10-12	September	5	2	0	0	13	0	4	17
	September	15	7	4	1	10	0	2	13
	September	25	3	12	0	9	2	0	11
	December	15	0	19	6	3	4	1	14
8 -10	September	5	3	12	1	12	0	3	16
	September	15	5	14	1	10	0	2	13
	September	25	8	15	3	7	0	2	12
	December	15	2	21	1	7	1	3	12
8-14	September	5	6**	12**	2	41	0	20	63***
	September	15	16	22	2	39	0	7	48
	September	25	17NS	37***	5	30	2	5	42NS
	December	15	3***	64***	9	14	8	9	40NS

[a] Bulbs lifted and plants examined for symptoms in April, 1980. A total of 80 bulbs were in each treatment; 30 of 12-14cm, 20 of 10-12cm and 30 of 8-10cm.

[b] Emerged without sheath or leaf lesions.

[c] Includes leaves and sheathes of non-emerged plants.

[d] Includes sheath lesions, leaf lesions and neck rot, but excludes non-emerged plants without obvious symptoms.

* Significantly different from standard planting (15cm depth in September): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.01$; NS, not significant (>0.05)

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	September	15	4	4	0	19	0	3	22
	September	25	6	10	2	14	0	3	19
	December	15	1	24	2	4	3	5	14
10-12	September	5	2	0	0	13	0	4	17
	September	15	7	4	1	10	0	2	13
	September	25	3	12	0	9	2	0	11
	December	15	0	19	6	3	4	1	14
8 -10	September	5	3	12	1	12	0	3	16
	September	15	5	14	1	10	0	2	13
	September	25	8	15	3	7	0	2	12
	December	15	2	21	1	7	1	3	12
8-14	September	5	6**	12**	2	41	0	20	63***
	September	15	16	22	2	39	0	7	48
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CHAPTER 3

MECHANISMS OF RESISTANCE TO *BOTRYTIS* IN NARCISSUS BULBS

This chapter describes experiments designed to investigate the mechanisms by which narcissus bulbs resist infection by species of *Botrytis*. Microscopical observations were made on fungal development and the host's response. Bulb scales were then examined for the presence of preformed and induced antifungal chemicals. A novel group of phytoalexins was identified and the possibility of a structural basis for their activity was investigated.

1. Infection development

A. Germination and germ tube growth on leaf and bulb scale surfaces

In an attempt to elucidate the reasons for the failure of *B. narcissicola* and *B. cinerea* to cause spreading lesions following inoculation with conidia in SDW (Chapter 1) the growth and development of these fungi on tissue surfaces was examined and compared with fungal development in SDW on glass slides.

Development on bulb scales (cv. Unsurpassable) and on clean glass was investigated in November 1977, and on bulb scales and leaves (cv. Golden Harvest) in May 1978. The influence of adding pollen to inoculum droplets on leaves (10^5 grains/ml) was also studied. Germination and germ tube lengths were assessed 6, 9, 12 and 24h after inoculation (Tables 3.1 and 3.2). The inability of conidia of

TABLE 3.1 *Germination of* B. narcissicola *and* B. cinerea *conidia on glass slides and leaves and bulb scales of narcissus*

Experiment	Surface	Species	% germination at intervals (h) after inoculation[a]			
			6	9	12	24
1. November 1977	Glass slide	*B. cinerea*	68 (58-78)	86 (84-88)	-[b]	90 (89-94)
		B. narcissicola	14 (7-20)	36 (31-42)	-	94 (90-98)
	Bulb scale[c]	*B cinerea*	36 (11-64)	58 (37-79)	79 (76-84)	81 (65-90
		B. narcissicola	39 (10-70)	72 (44-95)	98 (92-100)	96 (94-100)
2. May 1978	Bulb scale[d]	*B. cinerea*	94 (58-97)	93 (84-98)	93 (87-98)	>90
		B. narcissicola	36 (10-74)	92 (79-98)	69 (40-92)	>90
	Leaf[d]	*B. cinerea*	38 (17-59)	39 (32-46)	55 (40-70)	51 (34-68)
		B. narcissicola	4 (0-6)	27 (8-46)	40 (39-52)	47 (33-71)
	Leaf[d] (with pollen)	*B. cinerea*	80 (66-94)	94 (81-100)	>90	-
		B. narcissicola	94 (80-95)	92 (88-96)	>90	-

a Mean and range of replicate means.

b Not determined.

c Cultivar Unsurpassable.

d Cultivar Golden Harvest.

TABLE 3.2 Germ tube growth by B. narcissicola and B. cinerea on glass slides and leaves and bulb scales of narcissus

Experiment	Surface	Species	Germ tube length (µm) at intervals (h) after inoculation[a] 6	9	12	24
1. November 1977	Glass slide	B. cinerea	7 (3-10)	18 (14-22)	-[b]	21 (18-23)
		B. narcissicola	7 (3-10)	13 (11-16)	-	18 (16-19)
	Bulb scale[c]	B. cinerea	10 (4-15)	8 (5-11)	10 (4-21)	19 (5-44)
		B. narcissicola	13 (7-22)	38 (9-69)	32 (18-57)	>300
2. May 1978	Bulb scale[d]	B. cinerea	17 (3-33)	18 (5-41)	23 (7-41)	>300
		B. narcissicola	10 (7-15)	19 (6-27)	27 (16-40)	>150
	Leaf[d]	B. cinerea	10 (7-15)	15 (6-20)	27 (16-39)	25 (8-42)
		B. narcissicola	10 (3-18)	9 (4-13)	11 (8-14)	14 (10-19)
	Leaf[d] (with pollen)	B. cinerea	27 (15-39)	54 (22-85)	116 (82-152)	>300
		B. narcissicola	25 (13-36)	61 (36-78)	88 (61-104)	>300

a Mean and range of replicate means.

b Not determined.

c Cultivar Unsurpassable.

d Cultivar Golden Harvest.

B. narcissicola and *B. cinerea* to cause spreading lesions did not result from their failure to germinate. Germination and germ tube growth on bulb scales or leaves was usually better than on glass slides. When pollen was added to inoculum droplets on leaves, development of both species was rapid but only *B. narcissicola* caused spreading lesions. It was concluded from these observations that fungal colonisation of narcissus was restricted during or after penetration of bulb and leaf tissue. The development of infection hyphae was examined in more detail in bulbs.

B. Microscopical observations on the infection of bulb scales

Bulb scale epidermal strips were examined at daily intervals as symptoms developed. Strips from tissues inoculated with *B. narcissicola* and *B. cinerea* conidia or mycelium were observed. Death of host cells and hyphae was determined by the use of vital stains and by the absence of cytoplasmic streaming.

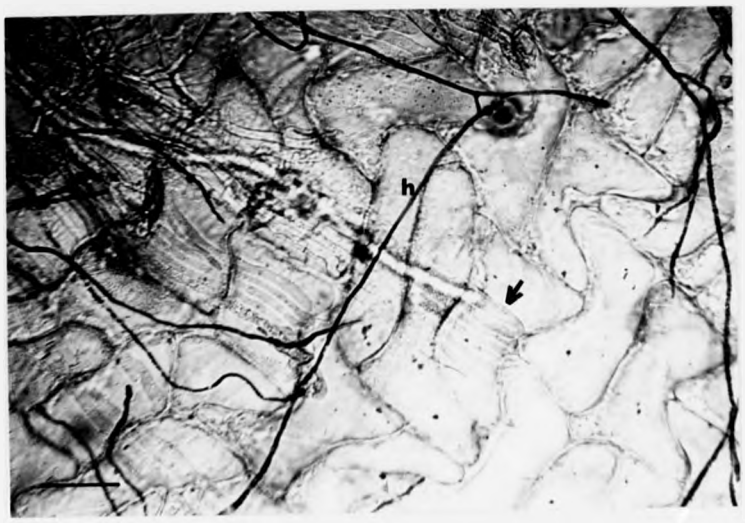
(i) Fungal development. Twenty-four hours after inoculation with conidia, both species appeared to have attempted to penetrate and to form infection hyphae. Tips of germ tubes were often swollen into a form of appressorium from which short infection hyphae had penetrated the underlying cell wall. Infection hyphae were often distorted, with granular contents. It was not possible to quantify differences between *B. narcissicola* and *B. cinerea*, in the time or frequency of penetration, because of the small numbers of successful penetrations and large variation between replicate strips.

Two days after inoculation with mycelium, successful infection by B. narcissicola was evident in the form of broad (c. 13µm) intracellular or intramural hyphae within epidermal strips (Plate 3.1). The hyphae radiated from a penetration point. Botrytis cinerea appeared to have attempted penetration; tips of hyphae were branched to form an infection cushion, but very few infection hyphae were observed (Plate 3.1).

(ii) Host response. The microscopial observation of B. narcissicola hyphae growing within the epidermis corresponded with the appearance of macroscopically visible spreading lesions. All colonised cells and some in advance of the invading hyphae were dead. Host cell death in resistant interactions (following inoculation with B. cinerea) was more restricted. Beneath B. cinerea mycelial inocula, a few isolated epidermal cells were killed (Plate 3.2). In epidermal strips from beneath conidial inocula small blocks of dead cells were commonly associated with sites of attempted penetration. Botrytis narcissicola tended to cause more extensive cell death than B. cinerea in resistant reactions, frequently killing a block of four or five cells beneath a tangle of granular hyphae. The observation of numerous dead cells paralleled the appearance of fleck lesions.

Twenty-four hours after inoculation of bulb scales with conidia of B. narcissicola or B. cinerea, or mycelium of B. cinerea, cell wall thickening and deposits of solid and granular reaction material were observed at sites of attempted penetration. The solid deposit (Plates 3.1 and 3.3) was commonly associated with spores producing short

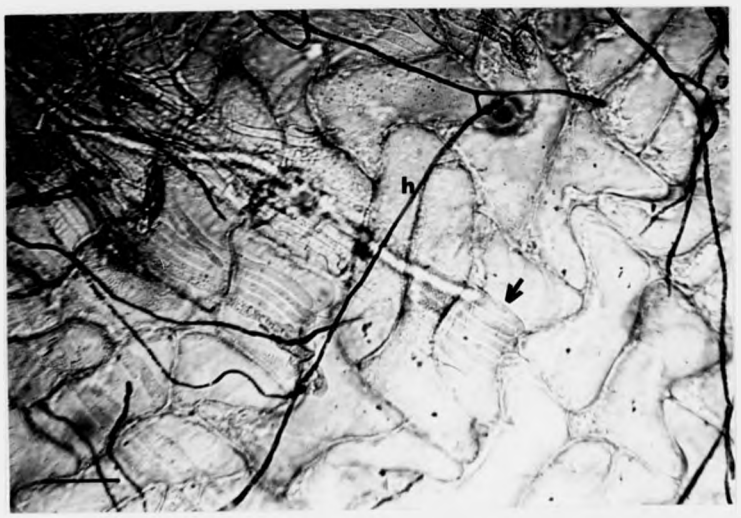
a



b

PLATE 3.1 Bulb scale epidermal strips 18h after inoculation of tissue with mycelium of B. narcissicola (a) or B. cinerea (b). In (a) note that the thin surface hyphae (h) have taken up the stain (toluidine blue) while the broad, intramural hyphae (arrowed) have not. In (b) note the deposit of reaction material (arrowed) beneath the multi-digitate hyphal tip (infection cusion, i.c.). Scale bar on (a) = 50µm and on (b) 20µm.

a



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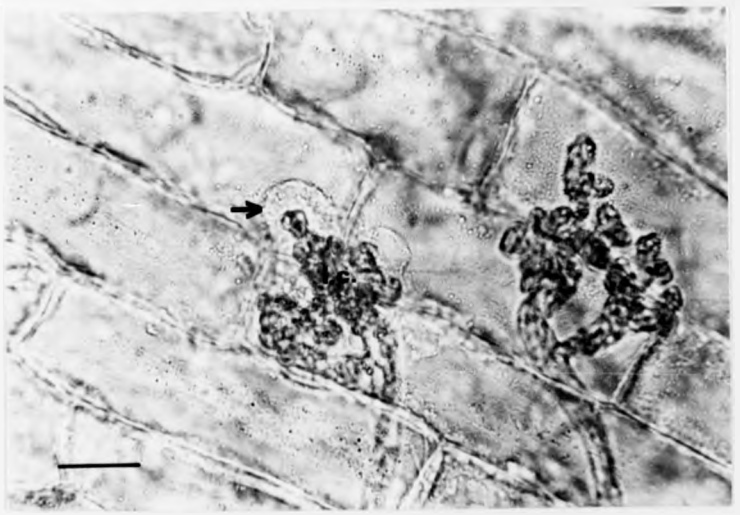

PLATE 3.1 Bulb scale epidermal strips 18h after inoculation of tissue with mycelium of B. narcissicola (a) or B. cinerea (b). In (a) note that the thin surface hyphae (h) have taken up the stain (toluidine blue) while the broad, intramural hyphae (arrowed) have not. In (b) note the deposit of reaction material (arrowed) beneath the multi-digitate hyphal tip (infection cusion, i.c.). Scale bar on (a) = 50µm and on (b) 20µm.

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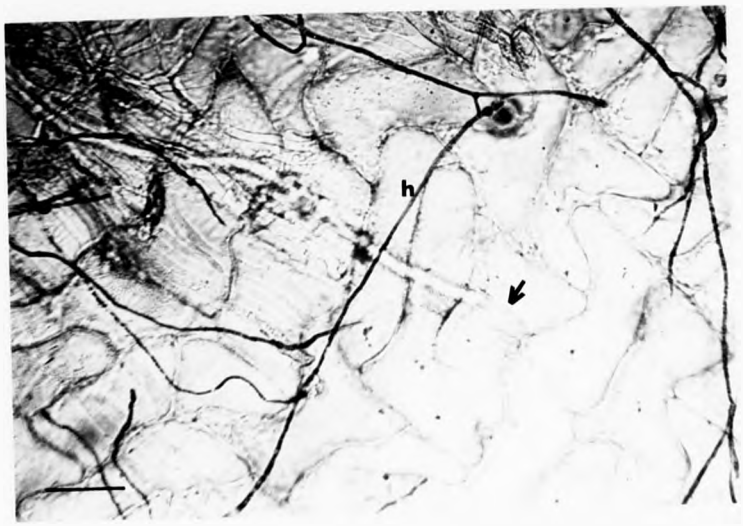

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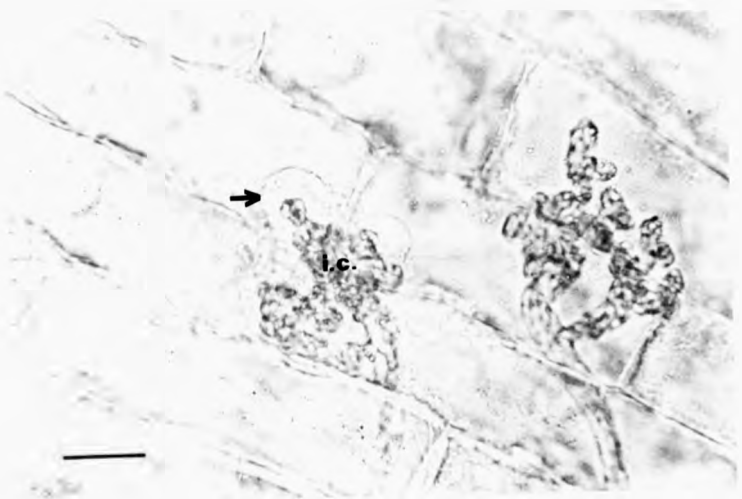


PLATE 3.1 Bulb scale epidermal strips 18h after inoculation of tissue with mycelium of B. narcissicola (a) or B. cinerea (b). In (a) note that the thin surface hyphae (h) have taken up the stain (toluidine blue) while the broad, intramural hyphae (arrowed) have not. In (b) note the deposit of reaction material (arrowed) beneath the multi-digitate hyphal tip (infection cusion, i.c.). Scale bar on (a) = 50µm and on (b) 20µm.

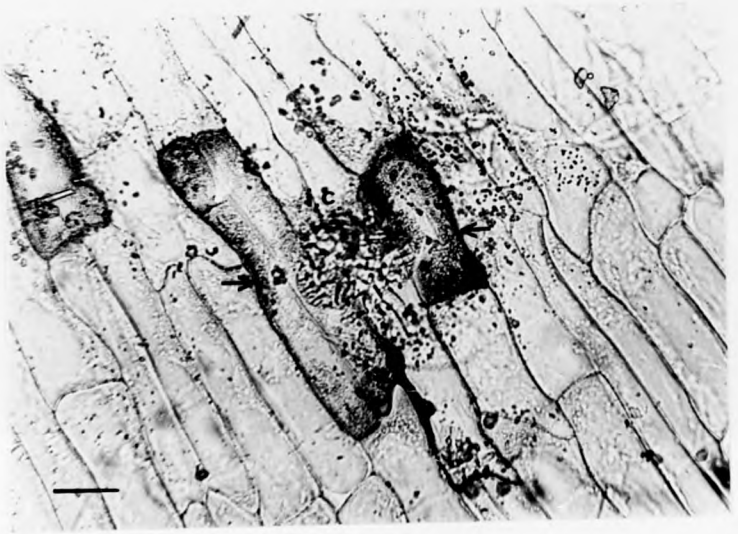


PLATE 3.2 Bulb scale epidermal strip 1 day after inoculation of tissue with mycelium of B. cinerea. Note the two dead cells with granular contents (arrowed) below the infection cushion (i.c.). Bar = 50μ,.

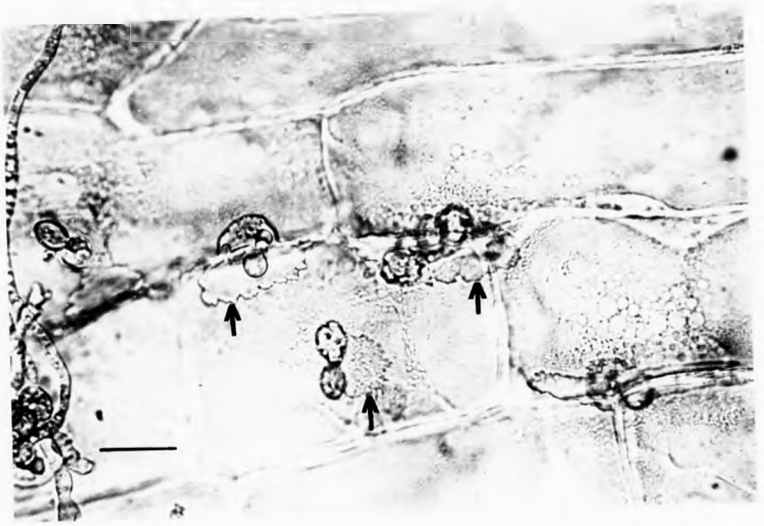
PLATE 3.2 Bulb scale epidermal strip 1 day after inoculation of tissue with mycelium of B. cinerea. Note the two dead cells with granular contents (arrowed) below the infection cushion (i.c.). Bar = 50µ.

a

b

PLATE 3.3 Bulb scale epidermal strips 2 days after inoculation of tissue with a conidial suspension of B. cinerea (a) or B. narcissicola (b). In (a) note the deposition of reaction material (arrowed) at sites of attempted penetration. In (b) a distorted infection hypha (arrowed) has been produced by B. narcissicola in spite of the deposition of solid reaction material(s) visible beneath the germ tube. Bar = 20µm.

a



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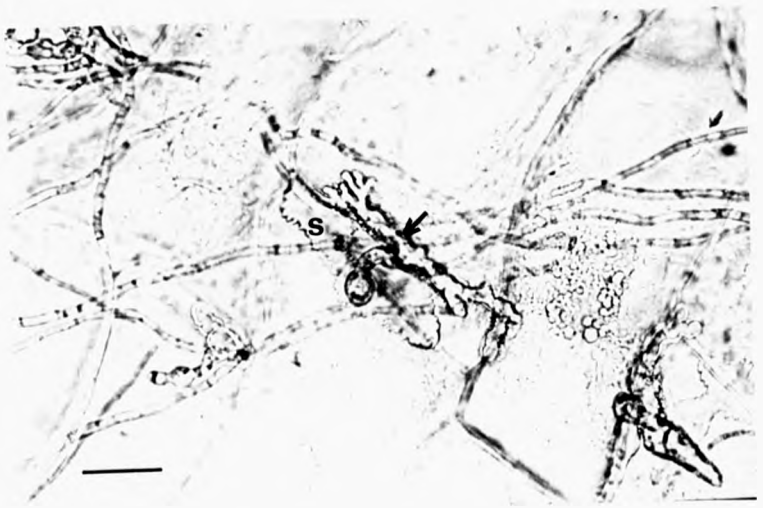

PLATE 3.3 Bulb scale epidermal strips 2 days after inoculation of tissue with a conidial suspension of B. cinerea (a) or B. narcissicola (b). In (a) note the deposition of reaction material (arrowed) at sites of attempted penetration. In (b) a distorted infection hypha (arrowed) has been produced by B. narcissicola in spite of the deposition of solid reaction material(s) visible beneath the germ tube. Bar = 20μm.

germ tubes (< 50µm) and with B. cinerea mycelial infection cushions. The granular deposit (Plates 3.4 and 3.5) was usually found adjacent to cell walls at sites of attempted penetration. During the first two days after inoculation reaction material was observed within living cells and following cell plasmolysis with sucrose the deposits were seen to be located between the retracted plasmalemma and the plant cell wall. Wall alterations were usually restricted to part of one wall (Plate 3.4) but occasionally all the walls of one cell, or a block of four or five cells, appeared to be thickened.

Because of the variation between replicate sites and the variety of responses observed, measurements of changes in frequency or extent of wall alteration or reaction material with time was not attempted. In general, however, beneath conidial inocula of both B. cinerea and B. narcissicola, and mycelial inocula of B. cinerea, a response in epidermal strips was first observed 1 day after inoculation and the number of sites at which material was present increased with time. Wall alterations and reaction material deposition initially occurred within living cells but many cells showing these responses died between 1 and 5 days after inoculation.

Reaction material and thickening of walls were rarely seen in epidermal strips invaded by B. narcissicola following mycelial inoculation and they were not observed in tissue beneath droplets of water.

The results of histochemical tests on bulb scale epidermal strips 24h after inoculation of tissue with

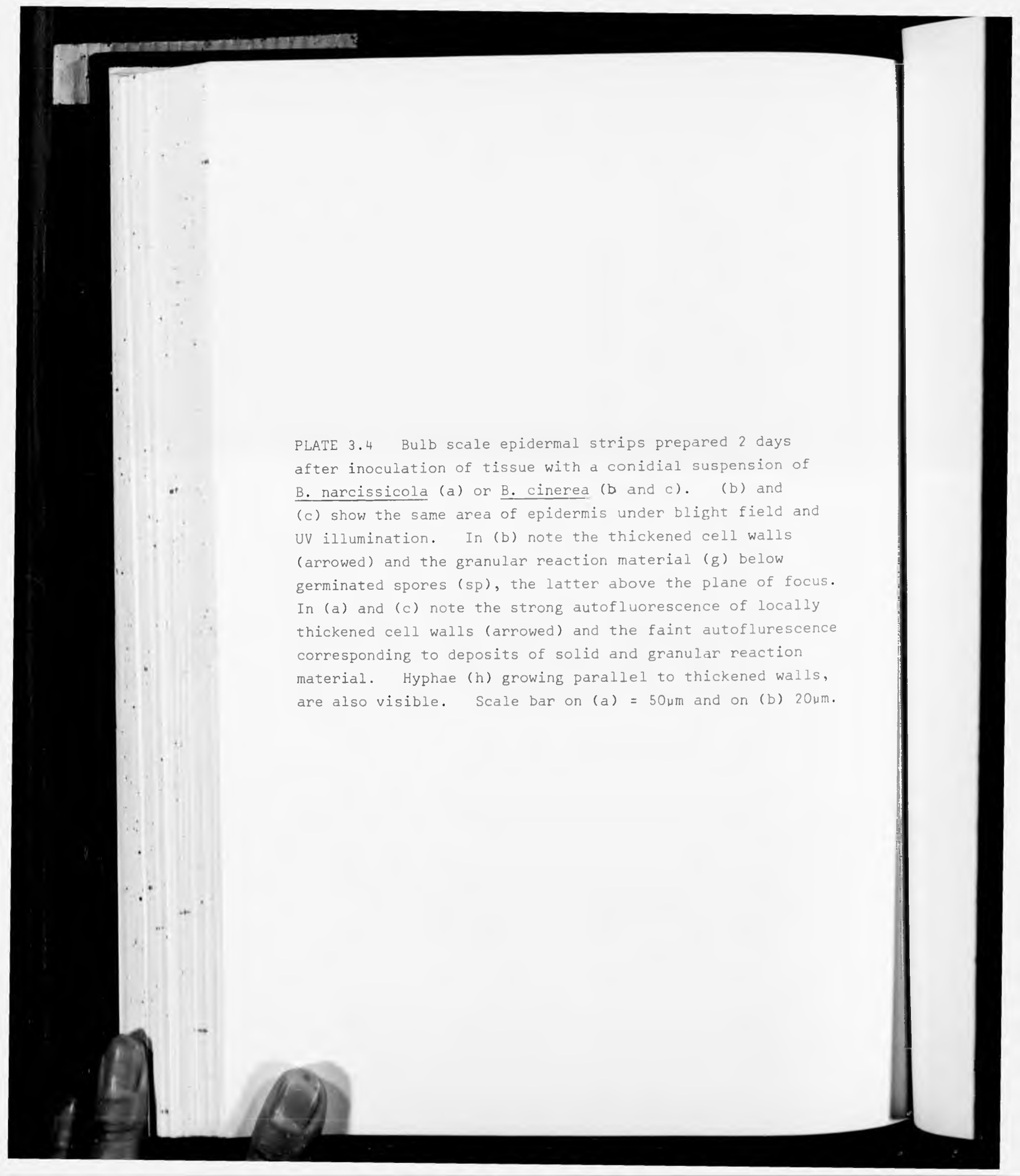

PLATE 3.4 Bulb scale epidermal strips prepared 2 days after inoculation of tissue with a conidial suspension of *B. narcissicola* (a) or *B. cinerea* (b and c). (b) and (c) show the same area of epidermis under blight field and UV illumination. In (b) note the thickened cell walls (arrowed) and the granular reaction material (g) below germinated spores (sp), the latter above the plane of focus. In (a) and (c) note the strong autofluorescence of locally thickened cell walls (arrowed) and the faint autoflurescence corresponding to deposits of solid and granular reaction material. Hyphae (h) growing parallel to thickened walls, are also visible. Scale bar on (a) = 50µm and on (b) 20µm.

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a

b

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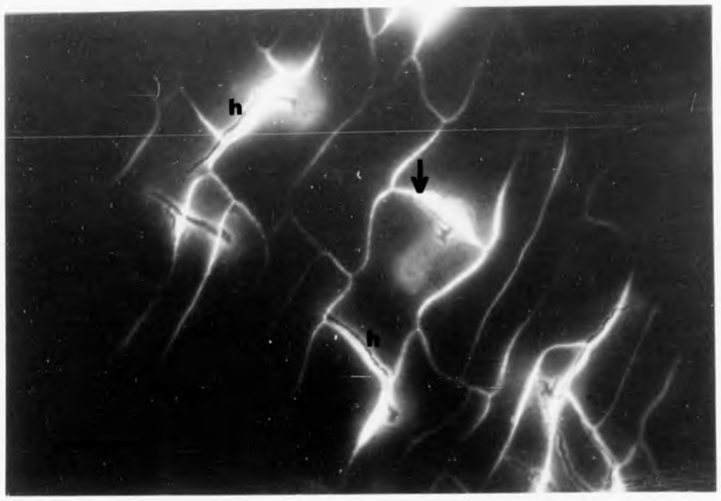
PLATE 3.4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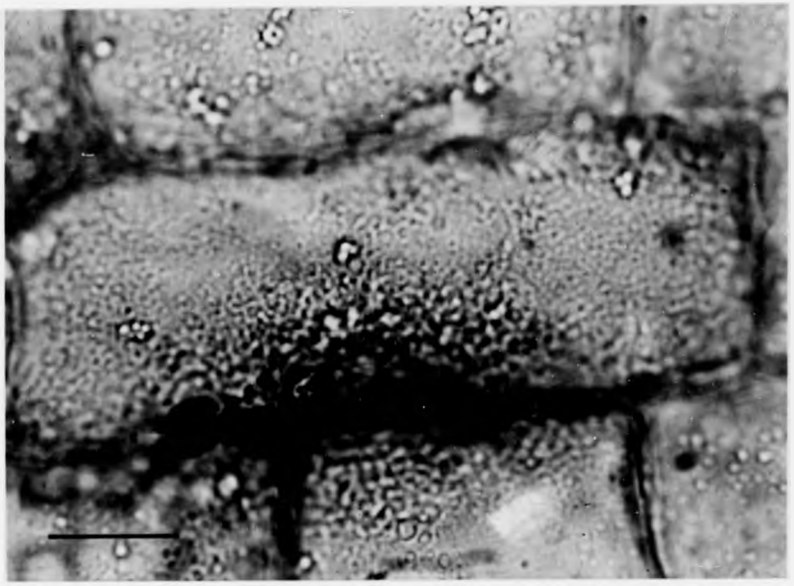
h


sp
g
sp

a



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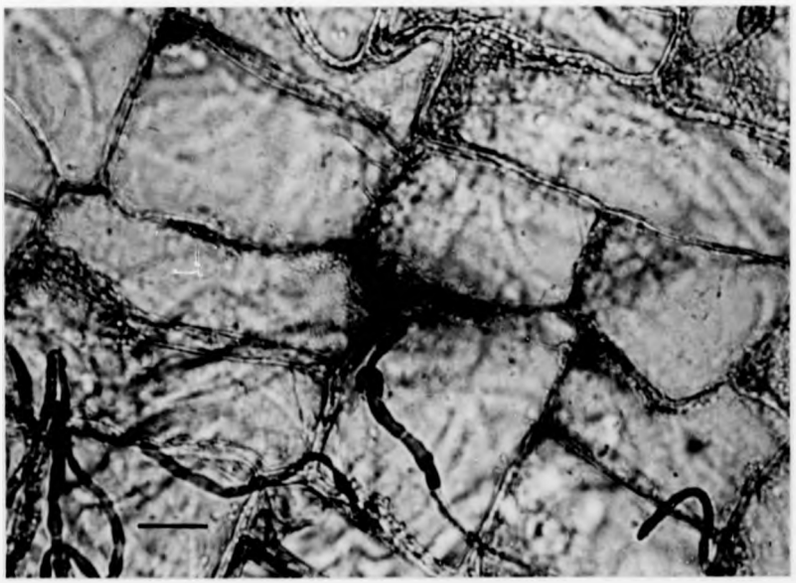
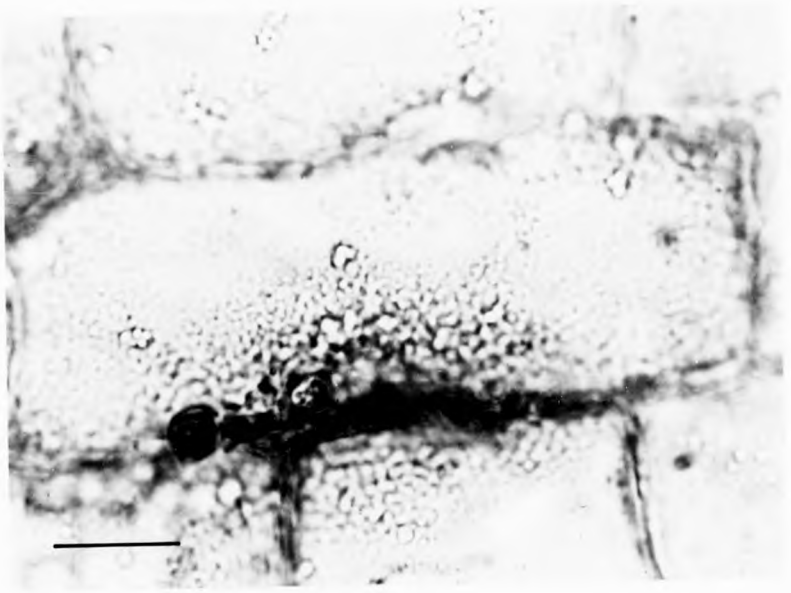


PLATE 3.5 Bulb scale epidermal strips 1 day after inoculation of tissues with conidia of B. cinerea (a) or B. narcissicola (b), stained with toluidine blue and azure B respectively. Note the intense staining of locally thickened walls at sites of attempted infection and lighter staining of granular material in the adjacent cytoplasm. Bar = 20µm.



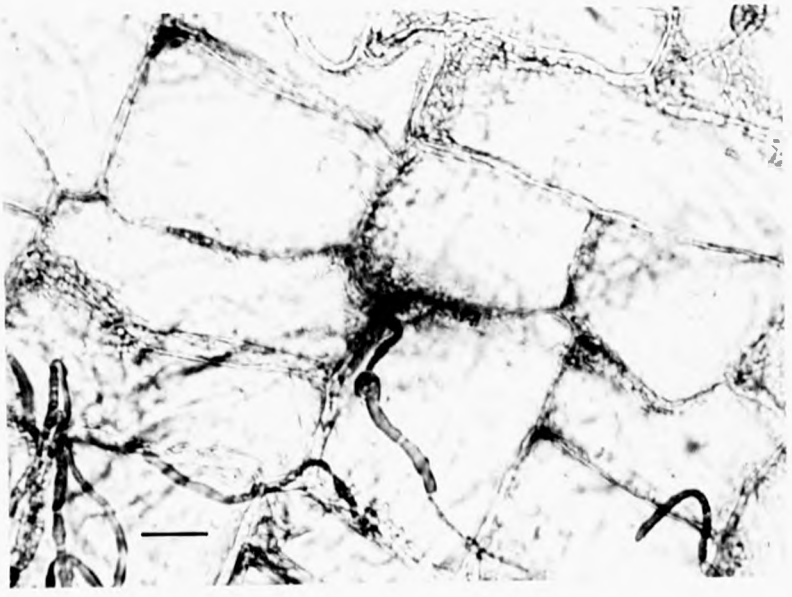


PLATE 3.5 Bulb scale epidermal strips 5 day after inoculation of tissues with conidia of B. cinerea (a) or B. narcissicola (b), stained with toluidine blue and mounted in water. Note the intense staining of locally thickened walls at sites of attempted infection and lighter staining of granular material in the adjacent cytoplasm. Bar = 20μm.

B. cinerea conidia are summarised in Table 3.3. Examination of epidermal strips following inoculation with conidia of *B. cinerea* or *B. narcissicola*, when observed under UV radiation, revealed a bright yellow autofluorescence of thickened cell walls and a faint autofluoroescence of adjacent reaction material (Plate 3.4). Fluoroescence was localised to sites of attempted penetration.

Several tests for lignin (azure B, toluidine blue, phloroglucinol/HCl) gave a positive result (Plate 3.5). Staining with alcoholic aniline blue but not with lacmoid indicated the presence of callose (Plate 3.6). The aniline blue fluorescence test for callose could not be used because of autofluorescence of unstained tissues. Locally thickened walls commonly gave a strong response to tests for lignin; the reaction material around germ tubes stained less readily. In most cases staining was associated with germinated spores or more obvious signs of attempted infection. In some epidermal strips a complete ring of dead cells, corresponding to the edge of a large but limited lesion, was found 3-5 days after inoculation.

The thickened cell walls observed in epidermal strips taken from beneath *B. cinerea* mycelial inocula also gave positive results to histochemical tests for lignin and callose. During spreading lesion development, bulb tissue generally did not take up these stains.

Microautoradiography provided further evidence that lignification was involved in the response to attempted infection. Radioactive material accumulated in localised deposits of EtOH insoluble phenolic polymers in epidermal strips following the injection of [3-^{14}C] cinnamic acid

TABLE 3.3 Histochemical tests on bulb scale epidermal strips 24h after inoculation with B. cinerea conidia

Test	Colour	Modified wall		Reaction material	
		Thin wall	Thick wall	Granular deposit	Solid deposit
For lignin					
UV fluorescence	Yellow	-	+	(±)	(+)
Phloroglucinol/HCl	Red	+	+	(±)	+
Toluidine blue 'O'	Blue/green	+	+	+	+
Azure 'B'	Green/blue	+	+	+	+
Chlorine/sulphite	-	-	-	-	-
For callose					
Aniline blue	Blue	+	+	-	-
Lacmoid	-	-	-	-	-

Key: + - stained.
- - not stained.
( ) - weakly stained.
± - variable.

a

b

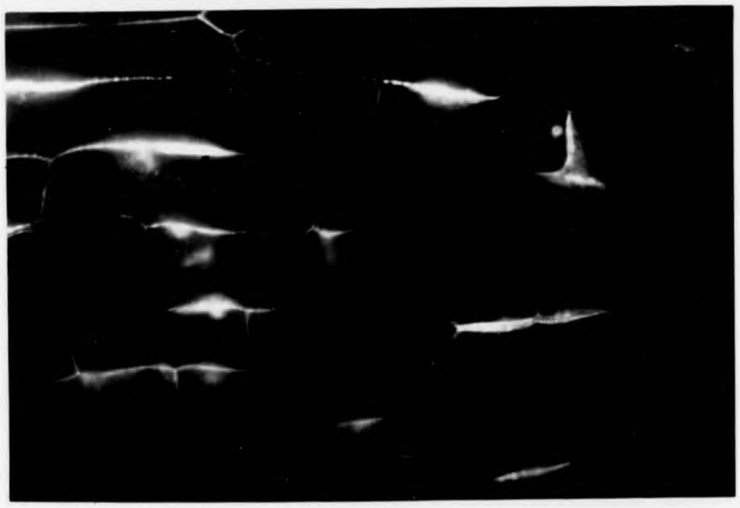
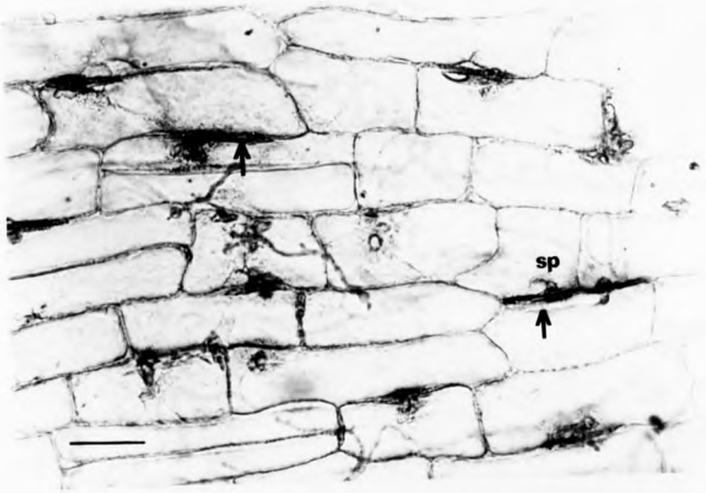


PLATE 3.6 Bulb scale epidermal strip mounted in alcoholic aniline blue, taken from tissue 2 days after inoculation with B. cinerea conidia. (a) Bright field illumination showing localised staining of cell walls (arrowed) below germinated spores (sp). (b) UV illumination of the same area. Note the strong fluorescence of locally thickened cell walls (arrowed) and the weaker fluorescence of adjacent cytoplasm. Bar = 50µm.

a



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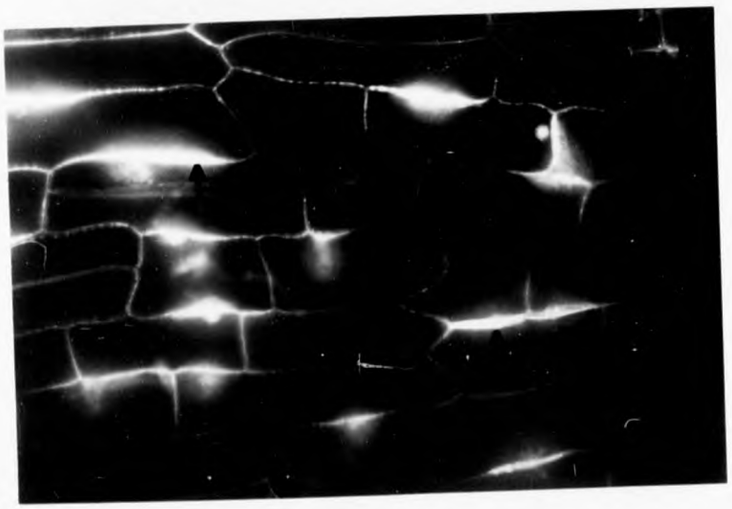

PLATE 3.6 Bulb scale epidermal strip mounted in alcoholic aniline blue, taken from tissue 7 days after inoculation with B. cinerea conidia. (a) Bright field illumination showing localised staining of cell walls (arrowed) below germinated spores (sp). (b) UV illumination of the same area. Note the strong fluorescence of locally thickened cell walls (arrowed) and the weaker fluorescence of adjacent cytoplasm. Bar = 50µm.

into bulb tissue beneath conidial inocula (Plate 3.7). No localised areas of high radioactivity occurred in epidermal strips beneath water inocula.

In conclusion, inoculations with B. cinerea or B. narcissicola conidia caused death of some host cells but tissue was invaded only following inoculation with B. narcissicola mycelium and this alone gave rise to spreading lesions. Infection development in all other interactions was restricted. Cell wall alterations and deposits of reaction material appeared to prevent penetration. Histochemical tests indicated the deposition of lignin or other polyphenolic material and possibly callose at sites of attempted penetration during the first day after inoculation.

2. Chemical inhibitors

Observations of distorted and granular infection hyphae within limited lesions suggested that chemical inhibitors were produced by narcissus bulb scales. Investigations were therefore made to see if a pre-formed inhibitor was present in healthy tissue or if phytoalexins accumulated in tissue undergoing a resistant response.

A. Search for a pre-formed inhibitor

(i) Invasion of frozen and leached tissue by B. narcissicola and B. cinerea. No difference in fungal growth on leached and non-leached frozen-thawed tissues was apparent 5 days after inoculation with mycelium of either species (Table 3.4). Botrytis cinerea failed to invade healthy tissue but grew rapidly through dead tissue; B. narcissicola developed a dense, sporulating mycelium on both leached

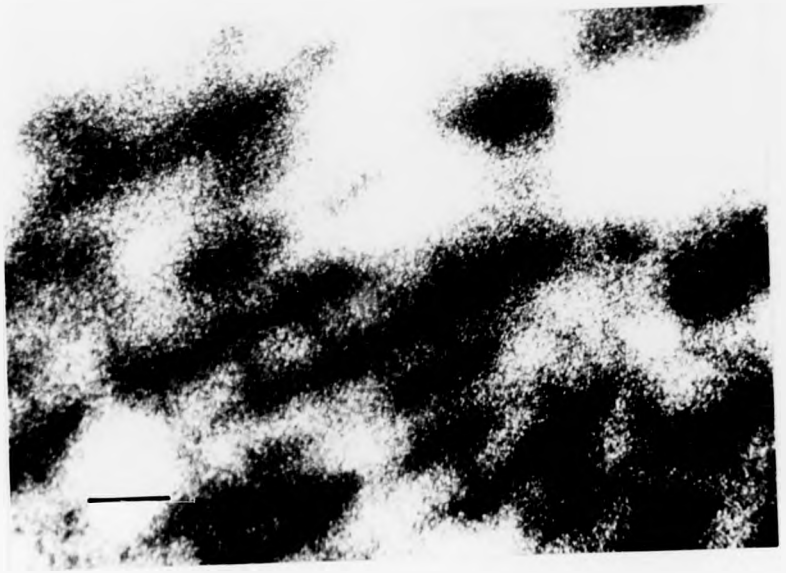

PLATE 3.7 Accumulation of radioactive material in an epidermal strip following injection of [3-^{14}C] cinnamic acid into bulb tissue beneath B. cinerea conidial inocula. The localised deposits of phenolic polymers were insoluble in EtOH. Bar = 50um.

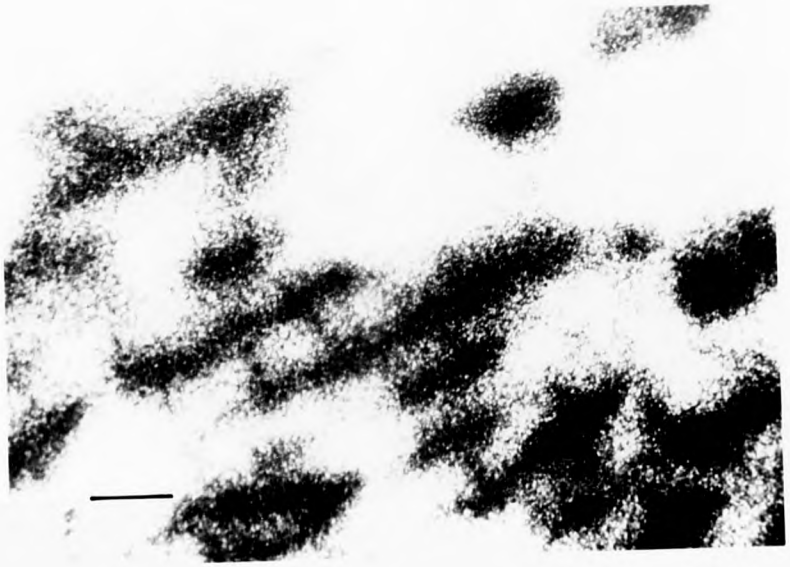

TABLE 3.4 Invasion of healthy, frozen/thawed and frozen/thawed/leached bulb scales, cv. Golden Harvest, by B. narcissicola and B. cinerea from mycelial inocula

Treatment	Invasion of bulb scales 5 days after inoculation[a]	
	B. narcissicola	B. cinerea
None (healthy scales)	++	-
Frozen/thawed	++++	+++
Frozen/thawed/leached	++++	+++

a Invasion was assessed by the extent and density of mycelium: -, no invasion; +, slight invasion; +++, major invasion; ++++, all scales rotted.

and unleached scales.

(ii) Bioassay of extracts from healthy (non-inoculated) tissue. A band of weak antifungal activity at R_F 0.77 - 0.87 (hexane-acetone 2:1) was detected in TLC plate bioassays of both Et_2O and MeOH extracts of non-inoculated bulb scales. No additional inhibitors were released by freezing and thawing the tissue. When epidermal and mesophyll tissues were collected separately the inhibitory band was detected in extracts of epidermal but not of mesophyll tissue.

Several zones of weak antifungal activity were detected in EtOAc and AmOH extracts of acid-hydrolysed epidermal and mesophyll bulb scale tissue by TLC plate bioassay (Table 3.5).

Although these results demonstrated the presence of a pre-formed chemical inhibitor (prohibitin) in bulb scale epidermis and the release of several antifungal compounds by hydrolysis, these inhibitors were only weakly active in the Cladosporium herbarum TLC plate bioassay. The rapid invasion of thawed tissues by B. narcissicola and B. cinerea indicates that preformed inhibitors probably do not contribute to the resistance of bulb scales to Botrytis.

B. Detection of phytoalexins

(i) In diffusates. The growth of B. cinerea in diffusates and after their sterilization by filtration, and in filtered diffusates extracted with Et_2O, is given in Table 3.6. In water diffusate (droplets incubated on bulb scales) growth of B. cinerea was similar to that in SDW. By

TABLE 3.5 Antifungal compounds extracted from non-inoculated, acid-hydrolysed bulb scale tissues, cv. Golden Harvest

Extraction solvent	Relative activity and R_F of antifungal zones[a]		Colour under[b] UV radiation (366nm)
	Epidermis	Mesophyll	
EtOAc	-[c]	+ (0 -0.05)	NV
	+[c] (0.04-0.16)	+ (0.06-0.16)	NV
	+ (0.24-0.34)	+ (0.23-0.34)	NV
AmOH	+ (0.47-0.54)	-	purple/brown

a Chromatograms were developed in hexane-acetone (2:1) for 15cm before spraying with C. herbarum spores.

b Developed chromatograms were examined under UV radiation, NV = not visible.

c -, no antifungal activity; +, slight antifungal activity; ++ moderate antifungal activity.

TABLE 3.6 Germination and germ tube growth of B. cinerea conidia in diffusates from bulb scales

Bioassay medium	Treatment	% germination[a]	Germ tube length[a] (µm)
Control (water)		80	60
Water diffusate (pH 5.0)	crude	82	108
	filtered	80	>500
	filtered/extracted	65	203
B. cinerea diffusate (pH 4.2)	crude	3	16
	filtered	41	70
	filtered/extracted	81	273

a Mean of three replicate droplets recorded after incubation for 24h.

contrast, the liquid from B. cinerea inoculum droplets after 24h incubation on bulb scales strongly inhibited germination and germ tube growth of B. cinerea conidia. Inhibitory activity was partly removed by filtration and completely removed with subsequent Et_2O extraction. The enhancement of germ tube growth by filtration of the water diffusate was reduced by subsequent Et_2O extraction.

These results indicated an accumulation of an Et_2O soluble inhibitor in B. cinerea inoculum droplets on bulb scales. The decrease in antifungal activity following filtration might result from the removal of bacteria.

(ii) In tissue. Growth of C. herbarum was markedly inhibited in a TLC plate bioassay of the Et_2O extract of tissue from beneath B. cinerea inocula, even at a concentration of only 0.05g fr.wt./ml (Plate 3.8). Four main bands of antifungal activity were resolved; R_F values in hexane-acetone (2:1) were 0.14-0.17, 0.26-0.33, 0.33-0.44 and 0.46-0.49. Similar bands, though only weakly antifungal, were present in Et_2O extracts of tissue collected from beneath water droplets (Plate 3.8). No antifungal activity was detected in any MeOH extracts of tissue remaining after Et_2O extraction.

3. Isolation and identification of phytoalexins

A. Preliminary investigations by TLC

(i) Number of phytoalexins accumulating. The Et_2O extract of tissue bearing limited lesions caused by B. cinerea, collected 3 days after inoculation, was chromatographed on TLC plates using a number of solvent

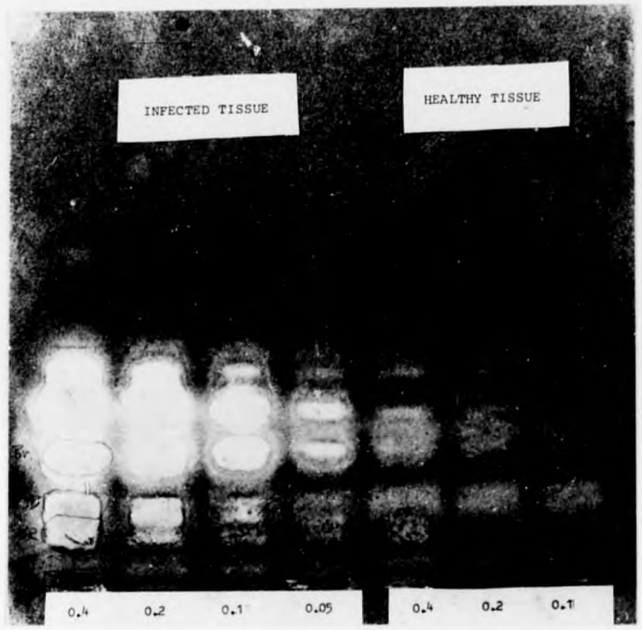

PLATE 3.8 TLC plate bioassay of the Et_2O extract from stripped bulb tissue (0.05 - 0.4g fr.wt.) collected 3 days after inoculation with SDW (healthy tissue) or a conidial suspension of B. cinerea (infected tissue). The chromatogram was developed in hexane-acetone (2:1).

systems. Ether-petroleum spirit (40-60°C) (2:1) proved the most successful, resolving seven inhibitory bands (Table 3.7).

With two-directional TLC, developing in Et_2O or Et_2O-petrol (2:1) in the first direction and hexane-acetone (2:1) in the second, nine antifungal zones were resolved when the extract from 0.1g fr.wt. of tissue was spotted at the origin and 12 when 1.0g fr.wt. of tissue extract was applied (Plate 3.9). The antifungal zones occurred in two groups, an upper diagonal of 6-8 zones, visible under UV radiation as quenching spots, and a lower diagonal of 3-4 zones, not visible under UV light. More than 20 UV-absorbing compounds were present in Et_2O extracts of bulb tissue.

A few weeks after developing chromatograms, some of the antifungal zones on the upper diagonal had become coloured (pink, yellow or orange).

(ii) Characterisation of phytoalexins with spray reagents. Three 20µl samples of an Et_2O extract were chromatographed in Et_2O-petrol (2:1) and bands visible under UV radiation were marked with a pencil. One chromatogram was sprayed with C. herbarum, a second with DpNA and the third with ethanolic $AlCl_3$; the two reagents are specific for phenolic and flavonoid compounds respectively.

Ten compounds developed a colour (yellow, pink or orange) with the DpNA spray and four became yellow with $AlCl_3$. From a comparison of R_F values, five of the DpNA positive compounds and two of the $AlCl_3$ positive compounds

TABLE 3.7 Separation of phytoalexins by TLC

Solvent system	Number of antifungal zones	R_F [a]	Antifungal activity [b]	Colour under UV radiation [c] (366nm)
Diethyl ether	2	0.69 - 0.84	++	-
		0.84 - 0.98	++	-
Chloroform	3	0 - 0.42	++	-
		0.71 - 0.78	+	-
		0.96 - 0.99	+	-
Hexane-acetone (2:1)	5	0.09 - 0.14	+	-
		0.14 - 0.17	++	-
		0.26 - 0.33	++	-
		0.34 - 0.44	++	-
		0.46 - 0.49	++	-
Ether-petrol (40-60°) (2:1)	7	0.09 - 0.11	+	NV
		0.13 - 0.17	++	P
		0.20 - 0.22	+	NV
		0.25 - 0.29	++	NV
		0.29 - 0.32	++	LP
		0.42 - 0.46	++	NV
		0.46 - 0.49	+	P

a Chromatograms developed twice (2x15cm).

b +, weakly antifungal; ++, strongly antifungal against *C. herbarum*.

c -, not recorded; NV, not visible; P, purple; LP, light purple.

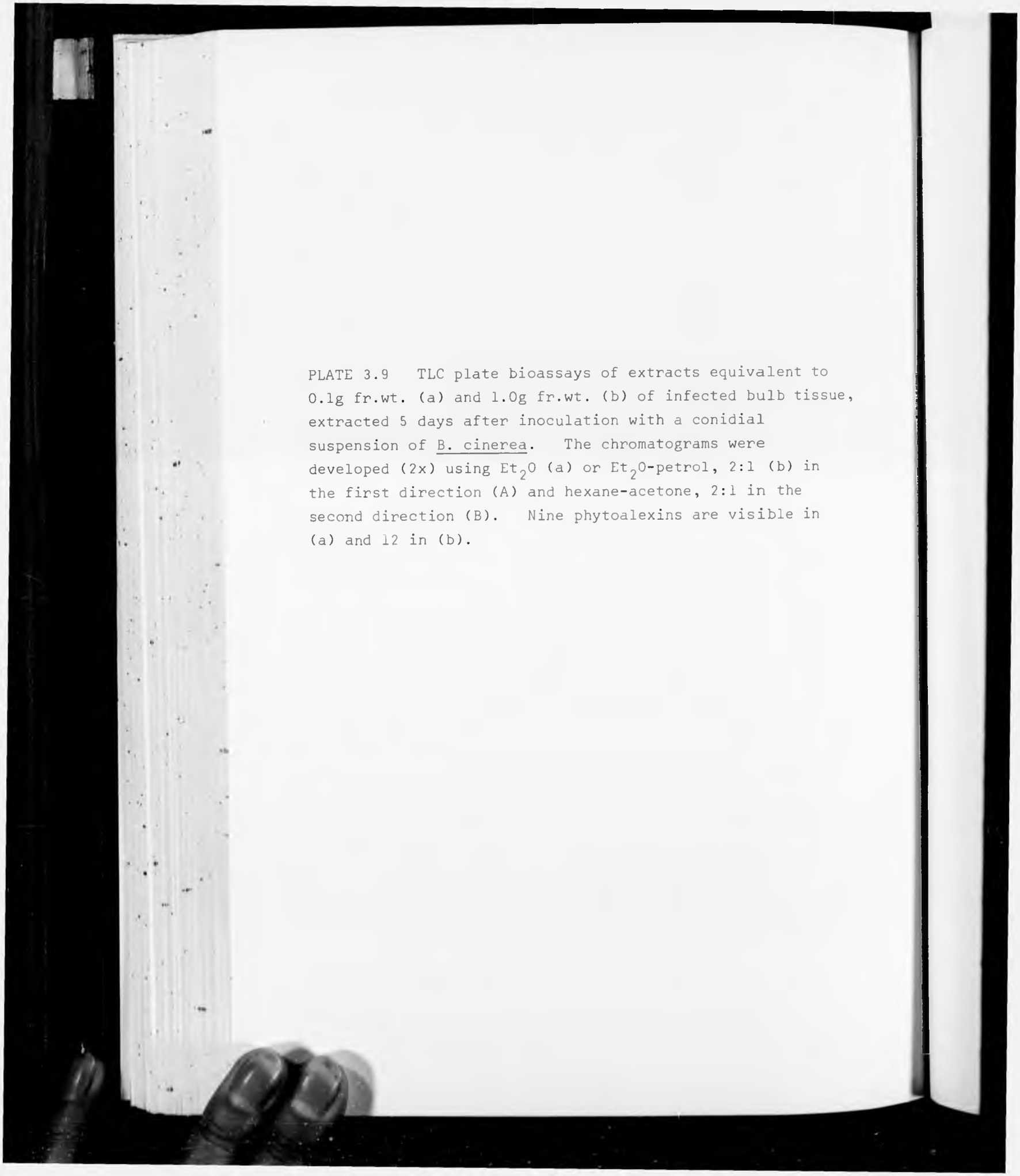

PLATE 3.9 TLC plate bioassays of extracts equivalent to 0.1g fr.wt. (a) and 1.0g fr.wt. (b) of infected bulb tissue, extracted 5 days after inoculation with a conidial suspension of B. cinerea. The chromatograms were developed (2x) using Et_2O (a) or Et_2O-petrol, 2:1 (b) in the first direction (A) and hexane-acetone, 2:1 in the second direction (B). Nine phytoalexins are visible in (a) and 12 in (b).

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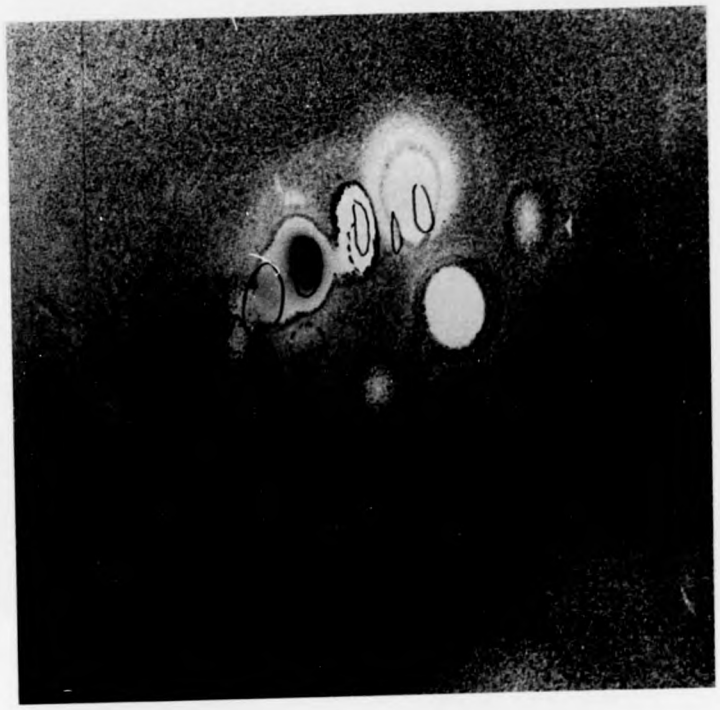

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PLATE 3.9

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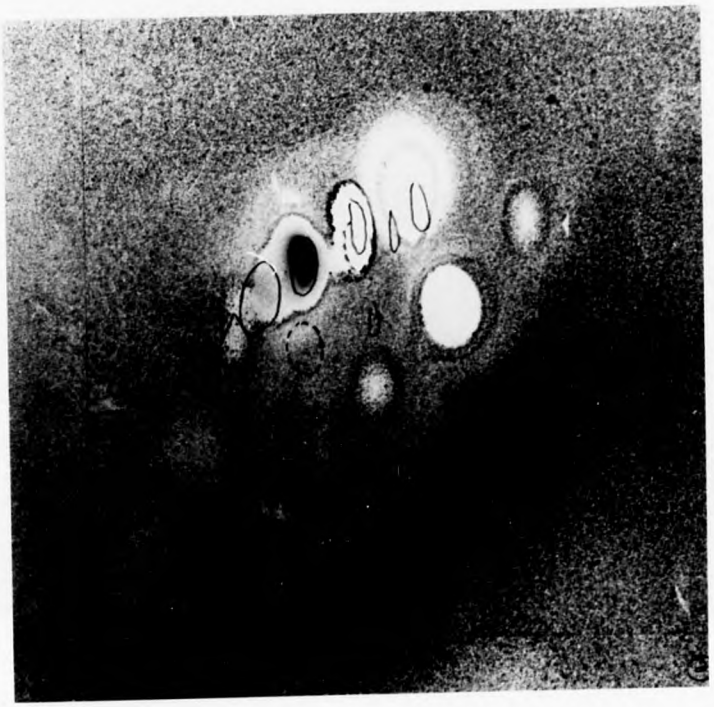

A

B

b

A

B

PLATE 3.9

(also DpNA positive) were found to be at positions of antifungal activity. A reference flavonoid compound, naringenin, gave a positive result to both tests but was not antifungal.

Thus, results showed that the response of narcissus to attempted Botrytis infection involves the production of at least 12 phytoalexins some of which are phenolic compounds, probably flavonoids.

B. Separation and purification of phytoalexins

(i) By gel filtration, TLC and HPLC. Phytoalexins from bulb scales were separated and purified for structural characterisation by a combination of gel filtration, TLC and HPLC (Fig. 3.1).

Initial fractionation of the crude Et_2O extract was by gel filtration. In the first experiment the extract from 40g fr.wt. of tissue was applied to the column and eluted at 2 ml/min. The phytoalexins eluted in two major groups, in fractions 15-16 and 25-31 (Fig. 3.2). Slight antifungal activity detected in fractions 37-38 was not investigated further.

TLC plate bioassay revealed four phytoalexins in the early eluting group, designated PA1-4 according to their increasing mobility in Et_2O-petrol (2:1). These compounds were not visible on TLC plates, at the concentrations present, under UV radiation at either 254 or 366nm, but they were readily located by their hydrophobic character after spraying with water. It was concluded that they correspond to the lower diagonal of antifungal zones revealed in two-directional TLC (Plate 3.9). Phytoalexins 2 and 3 separated by gel

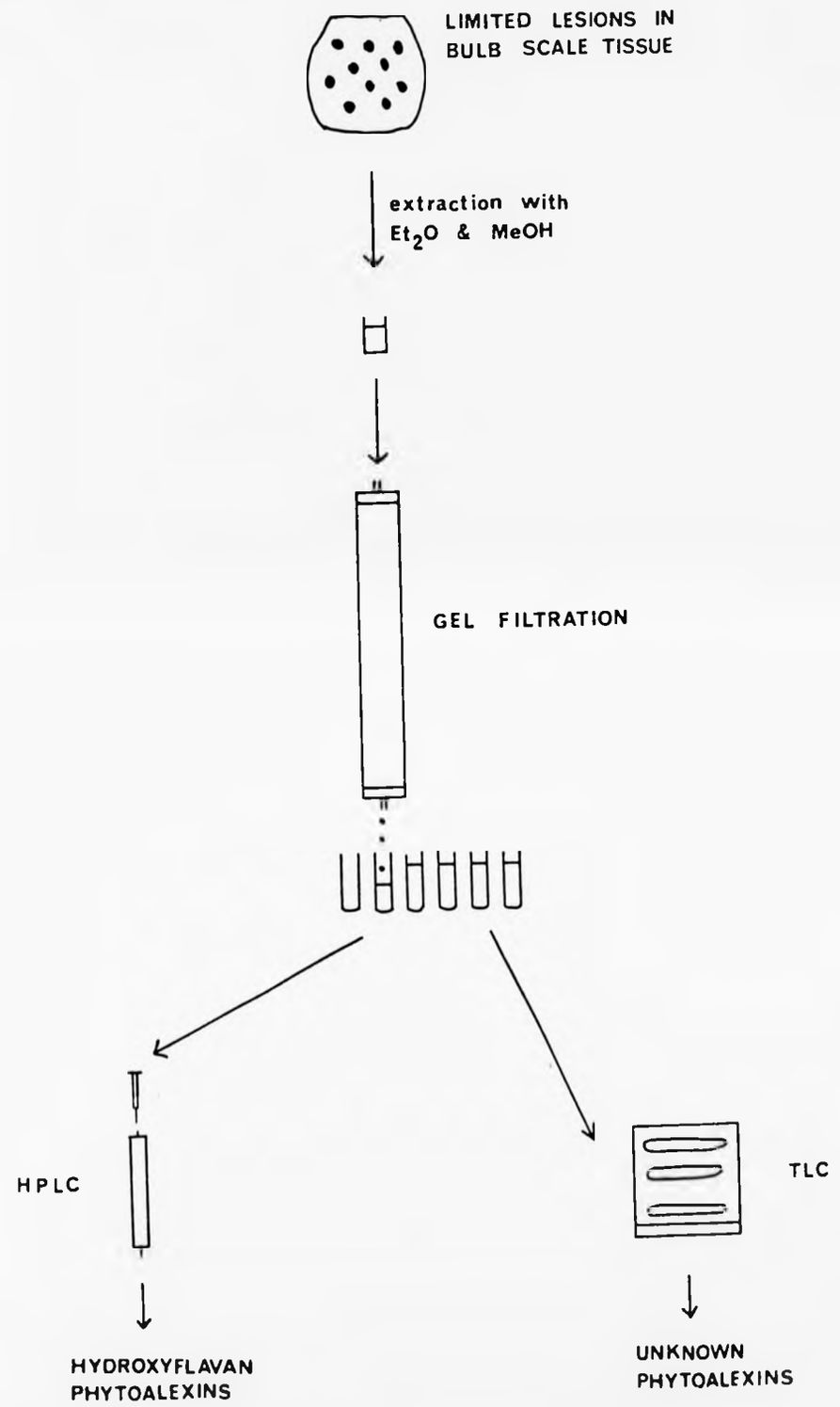

FIG. 3.1 Scheme for isolation of phytoalexins from infection bulb tissue by gel filtration, TLC and HPLC.

FIG. 3.2 Separation of phytoalexins from an extract of infected bulb tissue by gel filtration. Tissue (100g) was collected 5 days after inoculation of stripped scales with B. cinerea conidia (10^5 spores/ml). The Et_2O extract of the tissue was taken to dryness and resuspended in 10ml MeOH. A 4ml aliquot was applied to a 70 x 2.5cm column of LH20 Sephadex and eluted with MeOH at 2ml/min. Fractionation was monitored by UV absorbance (254nm). Twenty ml fractions were collected and tested (20µl) for antifungal activity against C. herbarum. The number of phytoalexins in each fraction was determined from bioassays of thin layer chromatograms.

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FIG. 3.2

filtration but with their similar R_F values in Et_2O-petrol (2:1) (Plate 3.10) were probably jointly responsible for the large, middle antifungal zone in Plate 3.9a.

With the later eluting set of phytoalexins, TLC in Et_2O-petrol (2:1) resolved five antifungal compounds, designated PA5-9 (Plate 3.11). These compounds were visible as quenching bands under UV light (254nm) and it was concluded that they correspond to the upper diagonal of antifungal zones, containing the phenolic compounds, in Plate 3.9. Phytoalexin 7 was identified on TLC plates by its pink colour, appearing within a few weeks, as illustrated in Plate 3.11. The phytoalexins 5, 6, 8 and 9 were located according to R_F.

A second batch of infected tissue was fractionated by gel filtration as fractions from the first batch were exhausted in preliminary examinations. An extract from 60g fr.wt. of infected tissue was eluted at 1 ml/min. Phytoalexins 1-4 eluted in fractions 19-21 and PA5-9 in fraction 35-45. The distribution and relative antifungal activity of the phytoalexins within the two major groups is shown in Table 3.8; R_F values after chromatography in Et_2O-petrol (2:1); water repellancy and characteristic colours in UV and white light are also presented.

Growth of C. herbarum was most strongly inhibited by PA2, 6, 7, 8 and 9. The last four were present at high levels in more than one fraction. Growth of C. herbarum was inhibited moderately by PA1 and PA3 and the least by PA4 and PA5.

PA1 PA2 PA3 PA4 PA4

PLATE 3.10 TLC plate bioassay of non-phenolic phytoalexins (PA1-4) extracted from bulb tissue and purified by gel filtration and PC. The chromatogram was developed twice in Et_2O-petrol (2:1).

PA1 PA2 PA3 PA4 PA5

PLATE 3.10 TLC plate bioassay of non-phenolic phytoalexin (PA1-5) extracted from bulb tissue and purified by gel filtration at 4°C. The chromatogram was developed twice in Et_2O-petrol (2:1).

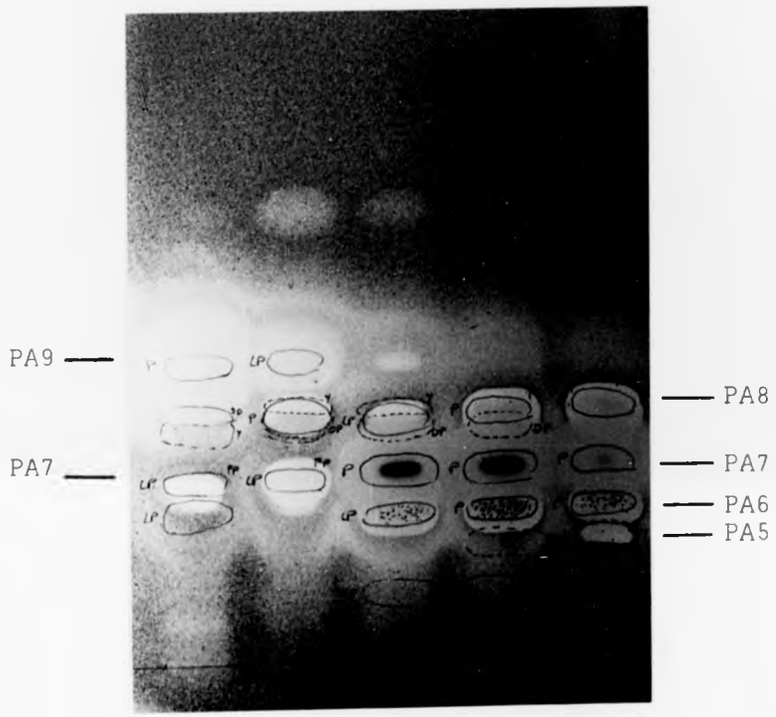

PLATE 3.11 TLC plate bioassay of selected antifungal fractions prepared by gel filtration (LH20 Sephadex) of an extract from limited lesions in bulb scales. Note that PA7 (7,4'dihydroxy-8-methylflavan) is visible as a dark red band where present at a high concentration.

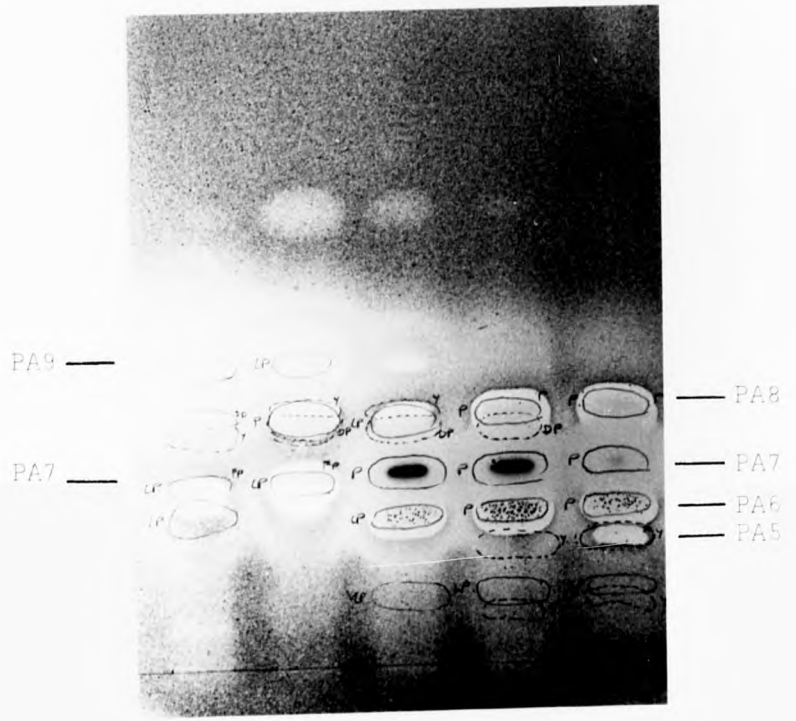

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TABLE 3.8 Separation of phytoalexins by gel filtration[a]

Phytoalexin	Antifungal activity in fraction[b]						R_F[c]	Colour[d]		Water repellency[e]
	20	36	38	40	42	44		366nm	WL	
PA1	++	-	-	-	-	-	0.09 - 0.18	NV	NV	+
PA2	+++	-	-	-	-	-	0.23 - 0.32	NV	NV	+
PA3	++	-	-	-	-	-	0.30 - 0.36	NV	NV	+
PA4	+	-	-	-	-	-	0.38 - 0.40	NV	NV	+
PA5	-	-	-	-	-	++	0.19 - 0.22	Y	NV	-
PA6	-	-	-	+++	+++	+++	0.22 - 0.30	DP	LY	-
PA7	-	+	++	+++	+++	++	0.30 - 0.36	P/Bl	Pi	-
PA8	-	-	+	+	++	+++	0.39 - 0.45	DP/R	O	-
PA9	-	+++	++	+	-	-	0.42 - 0.53	LP	NV	-

a See text for details.

b The first group of phytoalexins eluted in fractions 19-21 and the second group in fractions 35-45. Antifungal activity was determined by bioassay against C. herbarum; -, no inhibition; +, slight inhibition; ++, moderate inhibition; +++, strong inhibition. See also Plate 3.14 and Fig. 3.2.

c Chromatograms were developed in Et_2O-petrol (2:1). The R_F values listed are the lowest and highest limits found in chromatograms of either crude extracts or purified phytoalexins; widths of bands were usually less than indicated by these limits.

d Colour recorded under UV radiation (366nm) immediately or under white light (WL) after plates were exposed to the air for several weeks: NV, not visible; Y, yellow; DP, dark purple; P, purple; Bl, blue; R, red; LP, light purple; Pi, pink; LY, light yellow; O, orange.

e TLC plates were sprayed with water and then held up to the light: -, not repellent; +, repellent.

Phytoalexins 1, 2, 3, 6, 7, 8 and 9 were purified from selected fractions. Phytoalexins 1, 2 and 3 were purified from fraction 20 by PC in Et_2O-petrol (2:1) and subsequently hexane-acetone (2:1). Phytoalexin 9 was purified from fraction 36 and PA 6, 7 and 8 from fraction 42 by PC in Et_2O-petrol (2:1) (Plate 3.12). Amounts recovered from plates were c. 1mg (PA8), 2mg (PA2 and PA3), 4mg (PA6 and PA9) and 6mg (PA1 and PA7).

Phytoalexins 6-9 were further purified by HPLC. Retention times were 16-20, 22-30 and 64-72 mins for PA6, PA7 and PA9 respectively, following isocratic elution with 35% MeOH in 5% HCO_2H. Less than 0.1mg of PA8 was recovered, eluting after 46-48 mins.

The structures of purified PA1-PA3 and PA6-PA9 were investigated.

(ii) One-step HPLC fractionation of extracts. Preliminary work by R.V. Smallman (I.C.I. Ltd., Grangemouth) indicated that isocratic elution with 35% MeOH in 5% HCO_2H effected a reasonable one-step separation of crude extracts. The phenolic phytoalexins (PA6-9) with strong UV absorbance in the region 265-295nm (Figs 3.4-3.7) were suited to spectrophotometric detection. A wavelength of 284nm, λmax of PA6, was chosen. The non-phenolic phytoalexins (PA1-4), with weak UV absorbance at 284nm (Figs. 3.8-3.11), were not detected at this wavelength.

HPLC separation produced 38 UV absorbing peaks (Fig 3.3). Five fractions containing major peaks were strongly inhibitory (peaks 4, 24, 29, 34 and 37) and five weakly inhibitory (peaks 21, 22, 28, 30 and 35) to C. herbarum. Two

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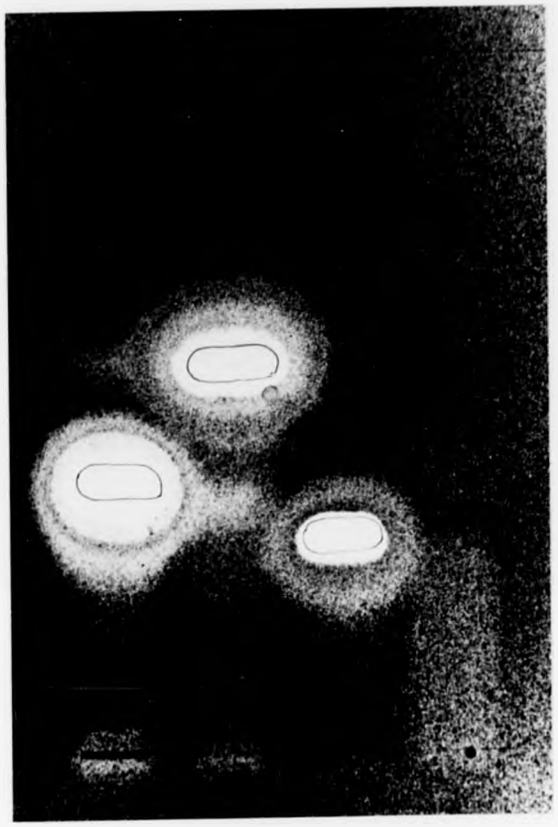
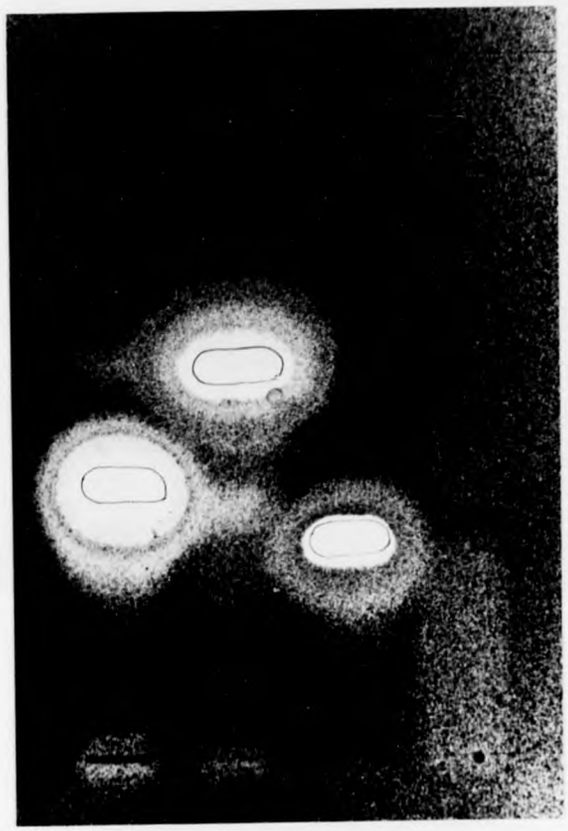


PA7 PA9 PA6 PA8

PLATE 3.12 TLC plate bioassay of phenolic phytoalexins extracted from bulb tissue after purification by gel filtration, PC and HPLC. The chromatogram was developed twice in Et_2O-petrol (2:1). PA7 is 7,4'-dihydroxy-8-methylflavan; PA9 is 7-hydroxyflavan; PA6 is 7,4'-dihydroxyflavan; PA8, an unidentified phenolic compound, was not antifungal at the low concentration tested.



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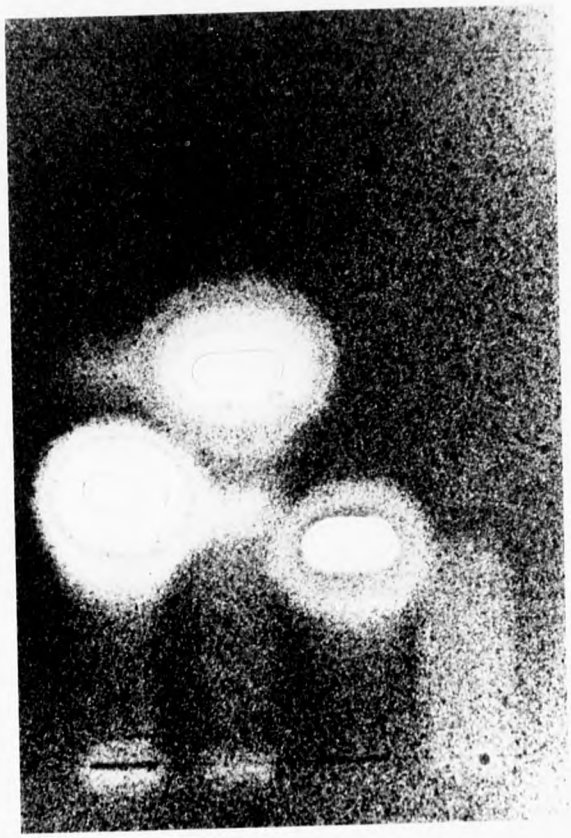

FIG. 3.3 Separation of compounds in an extract of infected bulb tissue by HPLC. Tissue (40g) was collected 5 days after inoculation of stripped scales with B. cinerea conidia (10^5 spores/ml). The Et_2O extract of the tissue was taken to dryness and resuspended in 1ml MeOH. A 25µl aliquot was injected onto a 5µm ODS Hypersil column (20x0.8cm) and eluted isocratically with 35% MeOH in 5% HCO_2H at 5ml/min. Elution was monitored by UV absorbance (284nm), 1.0 absorbance unit for full scale deflection (a.u.f.s.). Samples of each fraction (25µl) were spotted onto a TLC plate and assayed against C. herbarum. Peaks: 24 = 7,4'-dihydroxyflavan (PA6); 29 = 7,4'-dihydroxy-8-methylflavan (PA7); 34 = PA8 (unidentified) and 37 = 7-hydroxyflavan (PA9).

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FIG. 3.3

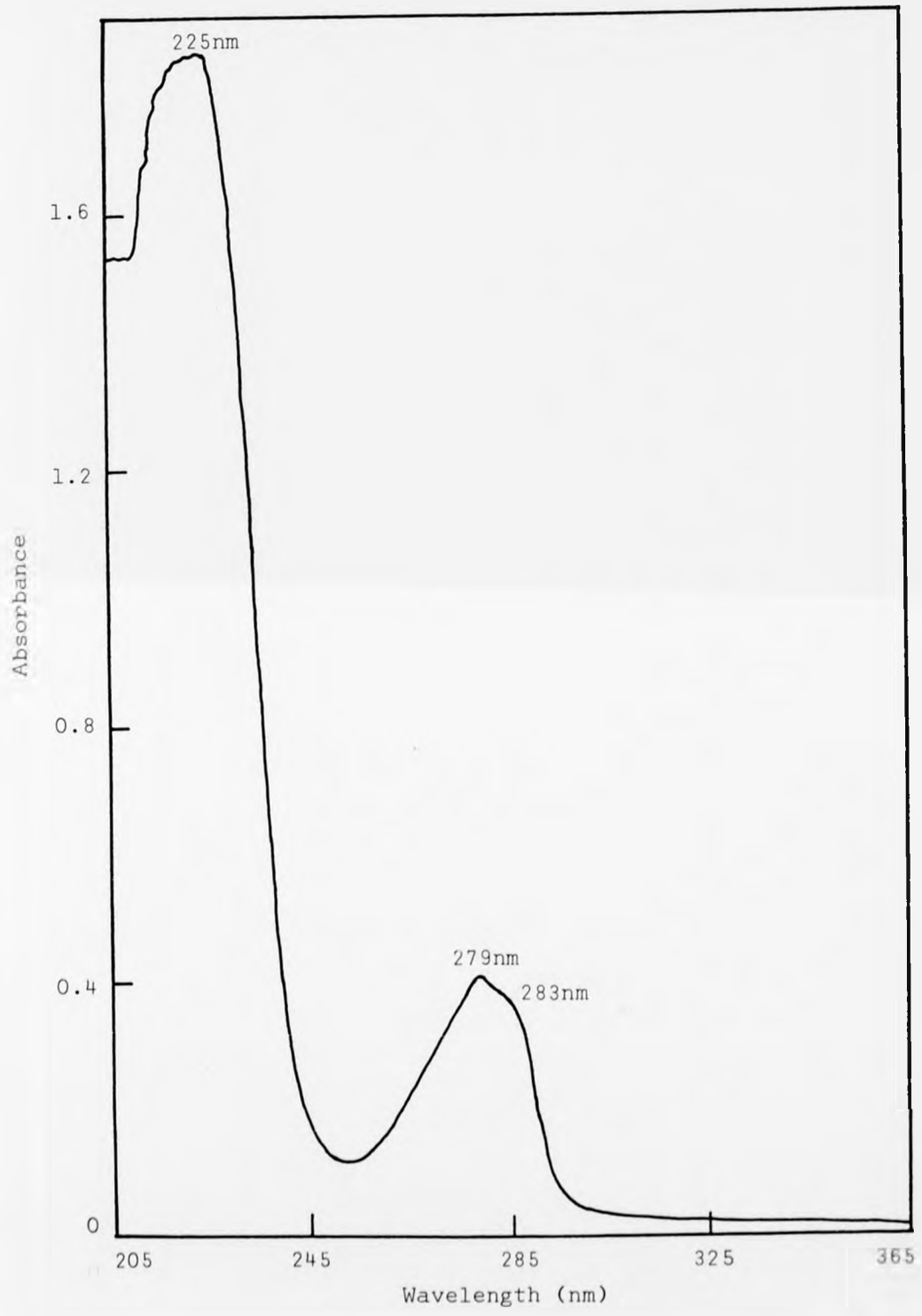


FIG. 3.4 UV absorption spectrum of 7,4'-dihydroxy-8-methylflavan (PA7) in MeOH.

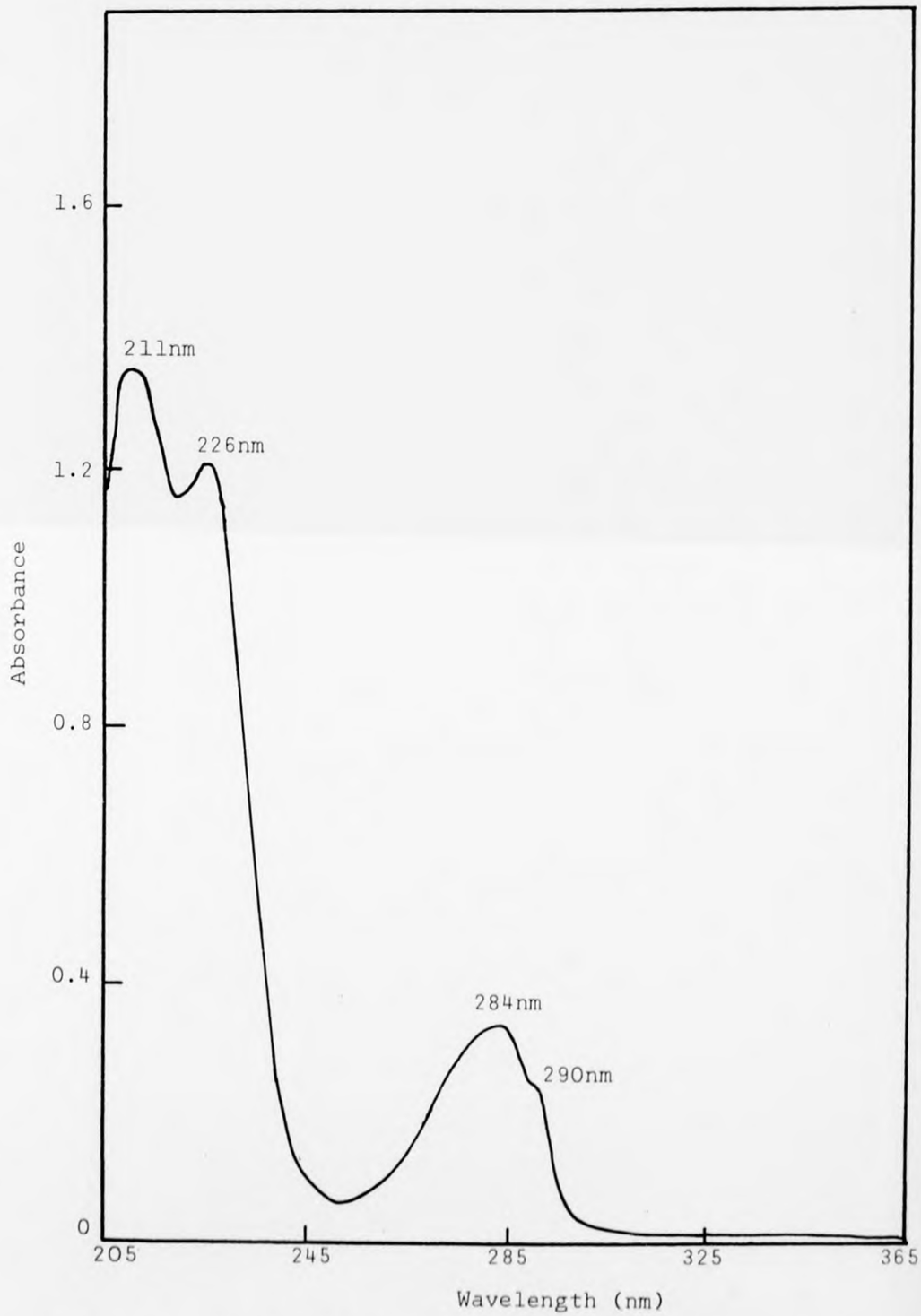


FIG. 3.5 UV absorption spectrum of 7,4'-dihydroxyflavan (PA6) in MeOH.

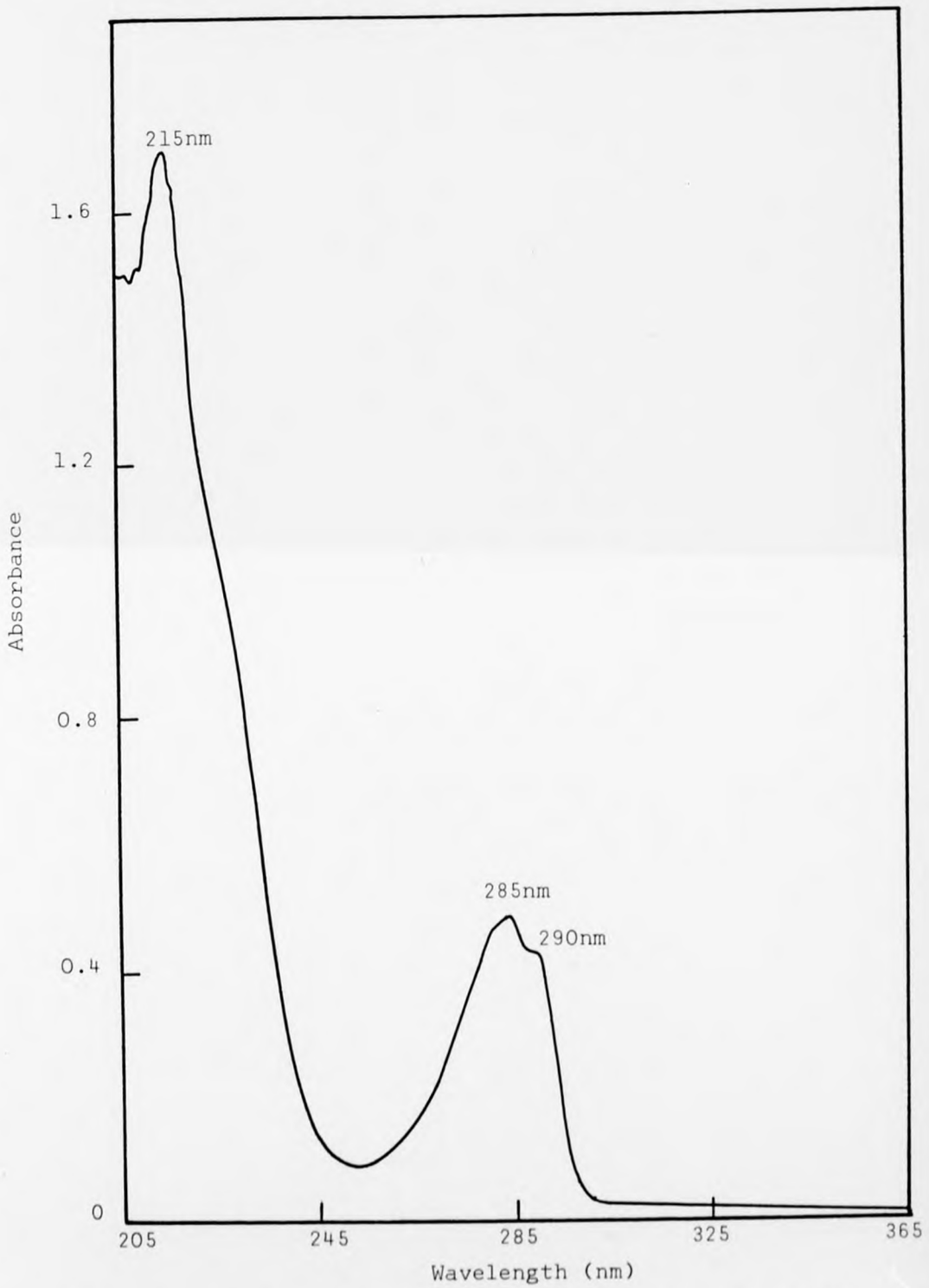


FIG. 3.6 UV absorption spectrum of 7-hydroxyflavan (PA9) in MeOH.

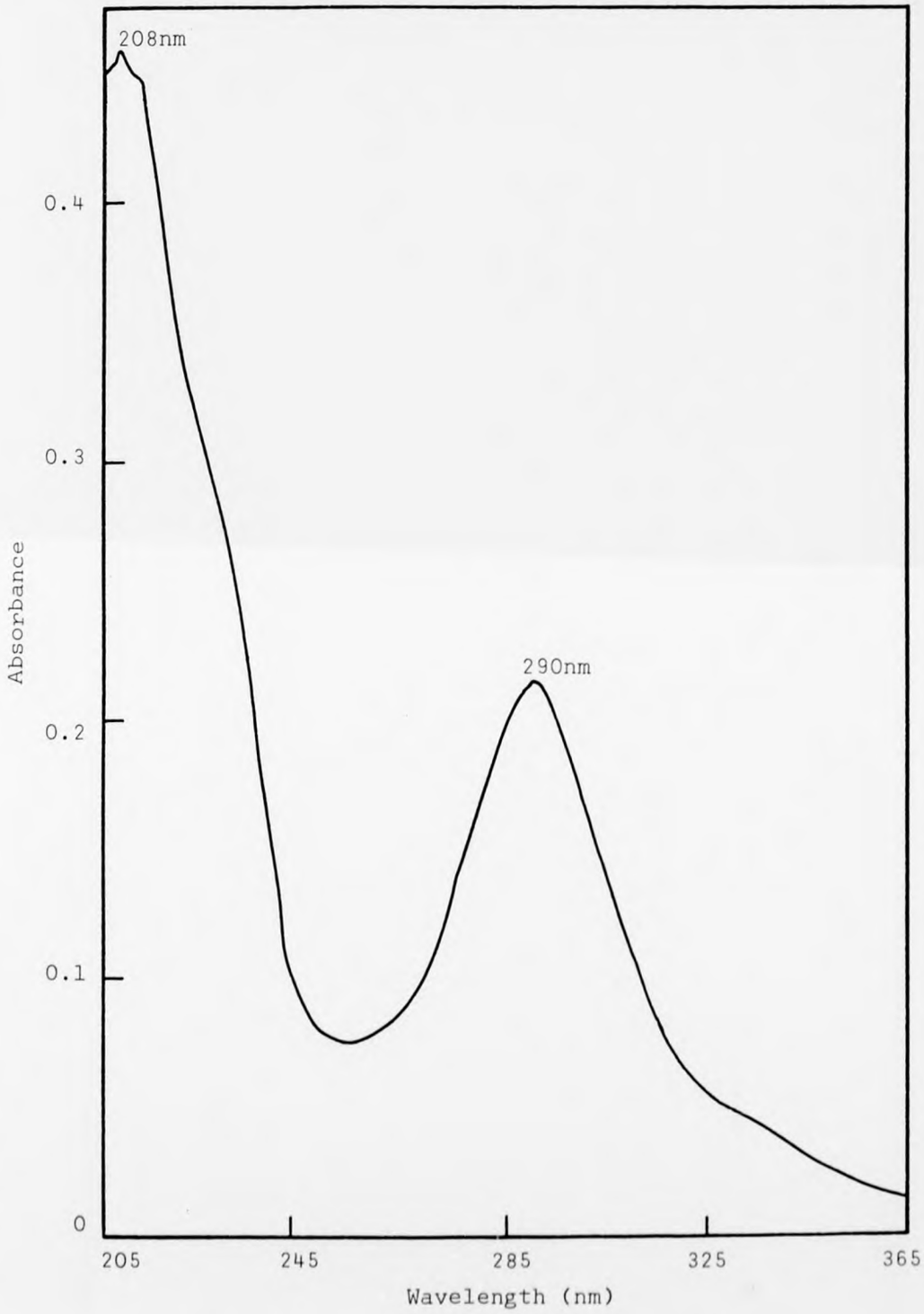


FIG. 3.7 UV absorption spectrum of PA8 in MeOH.

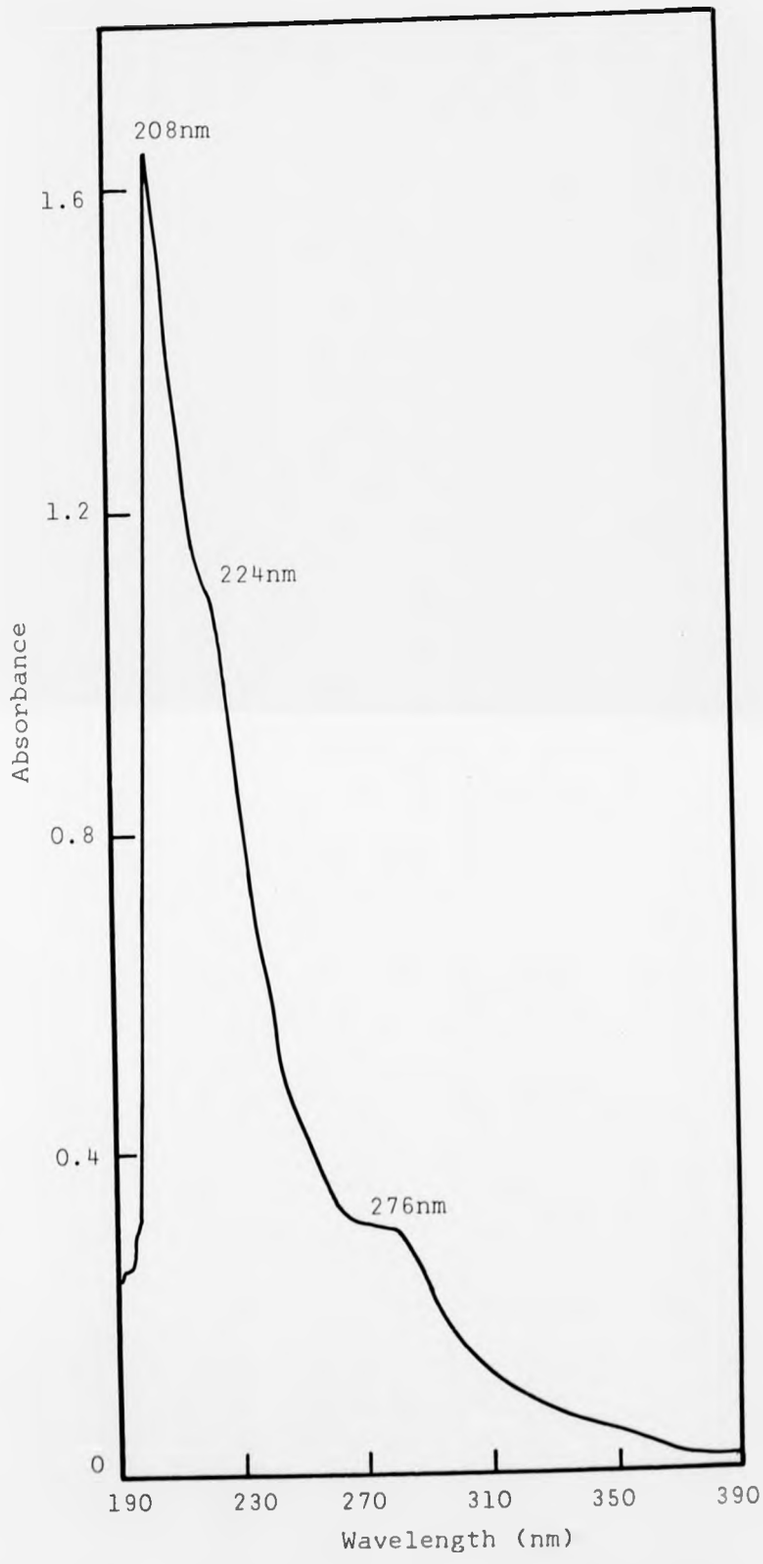


FIG. 3.8 UV absorption spectrum of PA1 in MeOH.

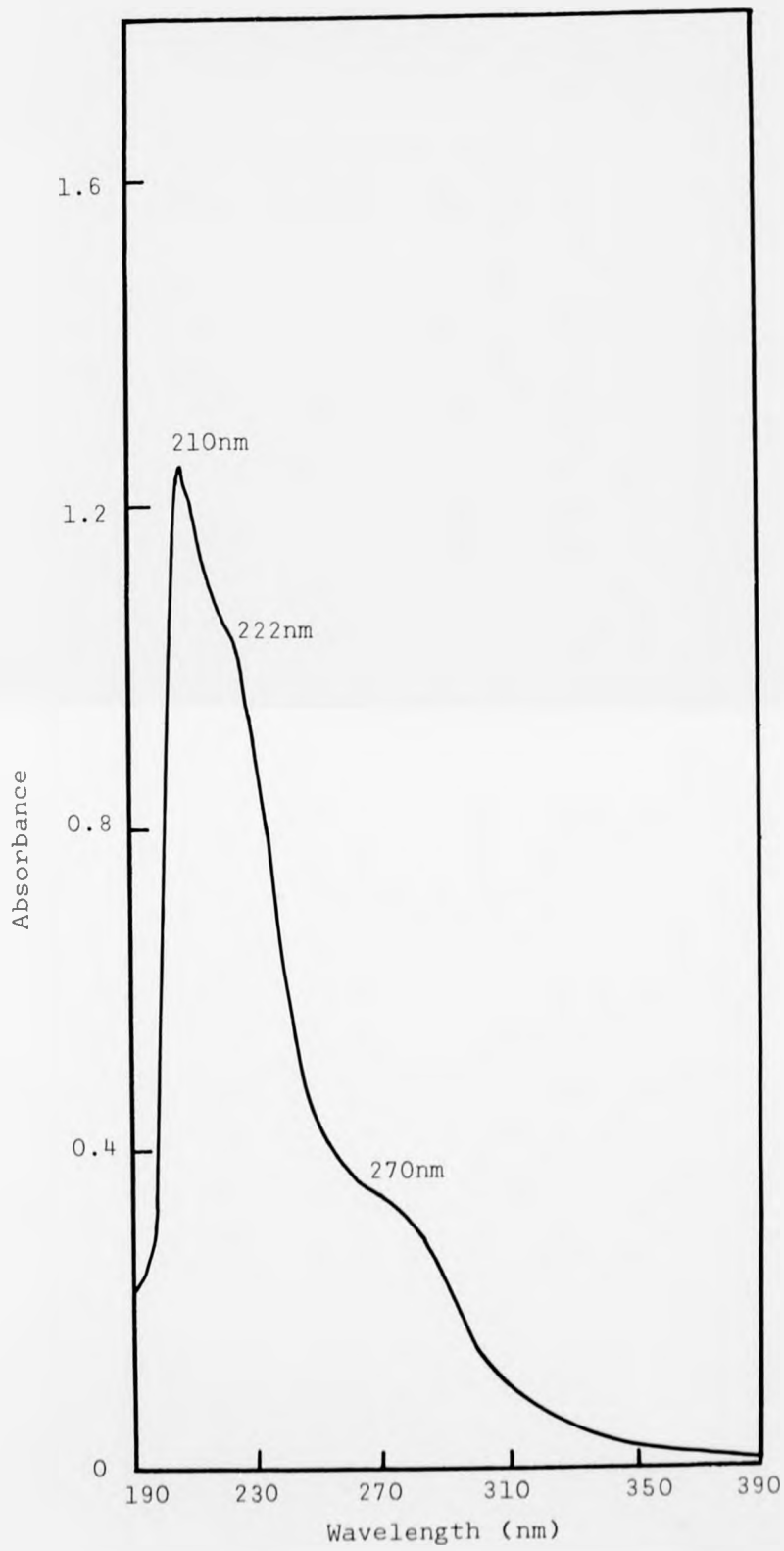


FIG. 3.9 UV absorption spectrum of PA2 in MeOH.

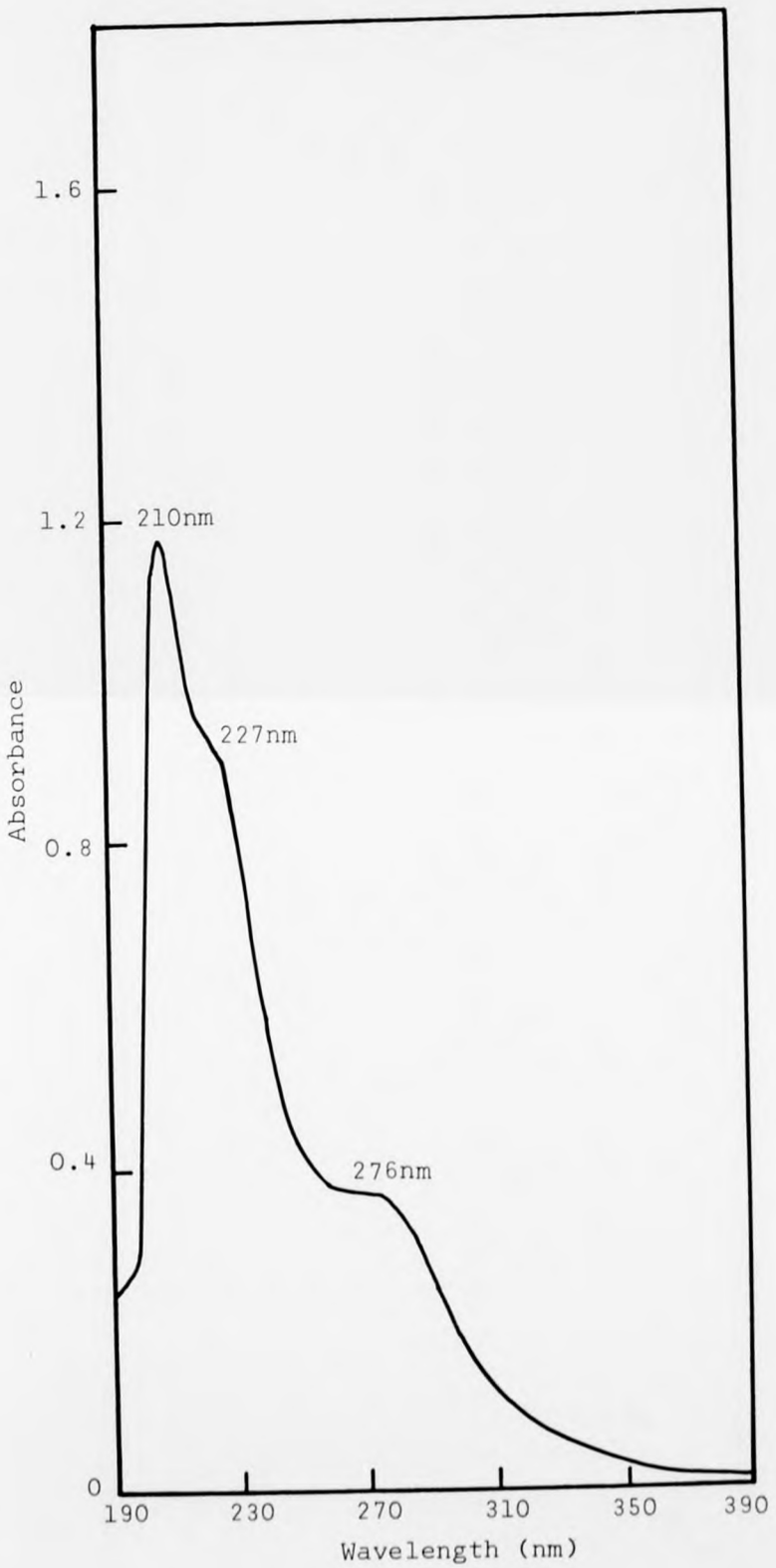


FIG. 3.10 UV absorption spectrum of PA3 in MeOH.

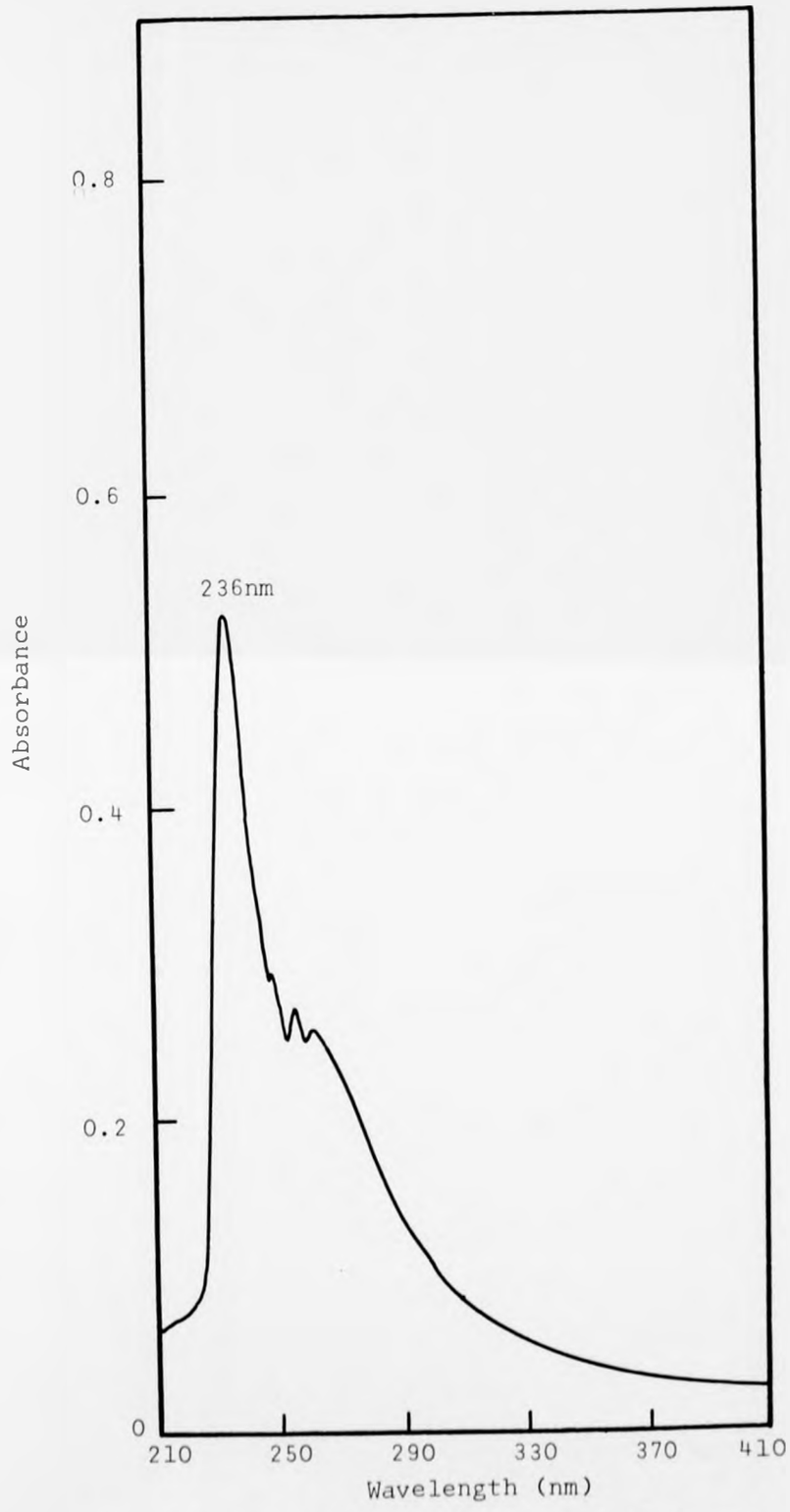


FIG. 3.11 UV absorption spectrum of PA1 in hexane.

fractions with no UV absorbance (fractions 28 and 33) were also strongly antifungal. Thus, a total of 12 phytoalexins was again indicated.

Activity of the fractions strongly antifungal to C. herbarum was confirmed by B. cinerea sporeling bioassay (Table 3.9), fraction 29 (peak 37) being the most active.

The identity of certain HPLC chromatogram peaks, deduced from R_F values in TLC and subsequently confirmed by co-chromatography with purified compounds, is shown in Table 3.9. Peaks 24, 29, 34 and 37 were identified as PA6-9 respectively.

C. Identification

(i) Phenolic phytoalexins. Purified PA6, PA7 and PA9 gave positive tests for phenols, being visualised on chromatograms by their yellow colour after spraying with DpNA. They were identified as 7-hydroxyflavan (PA9), 7,4'-dihydroxyflavan (PA6) and 7,4'-dihydroxy-8-methylflavan (PA7) (Fig. 3.12) from the following evidence.

High resolution mass spectrometry gave the following molecular weights and formulae: (PA9) M^+226.0989, $C_{15}H_{14}O_2$; (PA6) M^+ 242.0946, $C_{15}H_{14}O_3$ and (PA7) M^+256.1098, $C_{16}H_{16}O_3$. Molecular ions were present as base peaks in the mass spectra of all three compounds. Phytoalexin 7 was most readily characterised as (-)-7,4'-dihydroxy-8-methylflavan, $[\alpha]_D^{25}$-36.4° (C = 0.33, $CHCl_3$), by comparison of its physiochemical properties with those of the reported (-)-4'-hydroxy-7-methoxy-8-methylflavan (Cooke and Down, 1971). Prominent fragment ions in the mass spectrum of PA7

TABLE 3.9 Separation of phytoalexins by HPLC[a]

Antifungal fraction[b]	Peak Number	Phytoalexin	Retention time (mins)[d]	Inhibition of C. herbarum[d]	% inhibition of B. cinerea sporelings in SPN 0.1	0.5	1.0 g fr.wt./ml
1	1- 9	?PA5	0 - 3.6	+++	18	30	47
8	21		11.5 - 14.2	+	9	23	54
9	21		14.2 - 16.1	+	-[e]	-	-
11	22		17.2 - 18.8	+	9	38	48
12	23-24	PA6	18.8 - 21.8	+++	24	28	50
15	28		27.6 - 30.6	+	-	-	-
16	29	PA7	30.6 - 32.6	+++	39	99	100
17	29		32.6 - 36.0	+++	-	-	-
18	30-31		36.0 - 39.4	+	-	-	-
23	34	PA8	50.6 - 54.2	+++	36	60	100
25	35		55.8 - 59.6	+	23	98	99
28	No peak	?PA1-4	66.4 - 69.8	+++	-	-	-
29	37	PA9	69.8 - 73.4	+++	53	98	99
30	37		73.4 - 76.8	+++	-	-	-
33	No peak	?PA1-4	84.2 - 87.2	+++	14	22	41

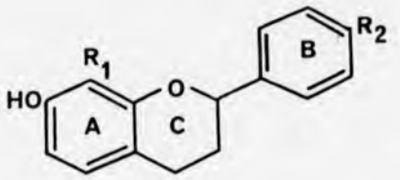
a See text and Fig. 3.3 for further details.

b All fractions were tested for antifungal activity by TLC plate spot bioassay; fractions not listed were inactive at the test concentration (0.1g fr.wt. tissue).

c A sample equivalent to 1.0g fr.wt. of infected tissue in 25µl MeOH was eluted isocratically with 35% MeOH in 5% HCO_2H.

d +, weak inhibition; +++, strong inhibition.

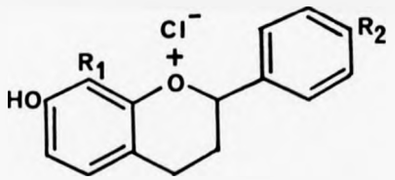
e Not determined.



$R_1 = R_2 = H$ 7-Hydroxyflavan (PA9)

$R_1 = H$, $R_2 = OH$ 7,4'-Dihydroxyflavan (PA6)

$R_1 = Me$, $R_2 = OH$ 7,4'-Dihydroxy-8-methylflavan (PA7)



$R_1 = R_2 = H$ 7-Hydroxyflavylium chloride

$R_1 = H$, $R_2 = OH$ 7,4'-Dihydroxyflavylium chloride

$R_1 = Me$, $R_2 = OH$ 7,4'-Dihydroxy-8-methylflavylium chloride

FIG. 3.12 Structure of hydroxyflavan phytoalexins and their flavylium salts.

were at m/e 137 ($C_8H_9O_2$, 85%) and 120 (C_8H_8O, 96%) arising from rings A and B respectively (Fig. 3.12), by retro-Diels-Alder (RDA) cleavage. The UV spectrum of PA7 (Fig. 3.4) exhibited maxima at 225, 279 and 283 sh nm (EtOH, log ε 4.27, 3.60, 3.59) differing only slightly from PA6 (Fig. 3.5) λmax 226, 284 and 290 sh nm (log ε 4.27, 3.67, 3.53) and PA9 (Fig. 3.6) λmax 211, 285 and 290 nm (log ε 4.41, 3.49, 3.43). The 90 MHz NMR spectrum of PA7 in methanol-d_4 showed six aromatic protons, four as an A_2B_2 quartet centred at δ6.80 and 7.26 and two as an AB quartet centred at 6.34 and 6.70. A singlet methyl signal at δ2.01 (2.51 in pyridine-d_5) was assigned to the methyl substituent in ring A. In pyridine-d_5 solution the protons in the heterocyclic ring were observed as a one-proton quartet at δ5.10 (J = 8 and 4Hz) assigned to the C-2 proton, a two-proton multiplet at 2.1 (C-3 protons) and a two-proton multiplet at 2.8 (C-4 protons).

Phytoalexin 6 lacked the aromatic methyl substituent in ring A. Its NMR spectrum was very similar to PA7 apart from changes associated with replacement of the methyl group by a proton. The mass spectrum of PA6 showed RDA fragments from ring A at m/e 123 (56%) and from ring B at m/e 120 (85%).

Phytoalexin 9 lacked the hydroxyl substituent in ring B but was otherwise identical to PA6. The difference was apparent in the aromatic region of the NMR spectrum and from the mass spectrum which gave a prominent RDA fragment at m/e 104 (46%) from ring B.

The hydroxyflavan structures proposed (Fig. 3.12) were confirmed by total synthesis. Flavylium salts (Fig. 3.12) were prepared by Robinson condensation of the appropriate hydroxybenzaldehydes with the corresponding acetophenones. Catalytic hydrogenation of the flavylium salts gave the racemic flavans. Full details of the syntheses are given in Coxon et al. (1980); physical properties of the purified compounds (m.p., MS, NMR) are also reported.

The chromatographic (TLC and HPLC) and spectral (MS, UV and NMR) properties of the natural and synthetic compounds were identical. The antifungal activity of the synthetic racemic hydroxyflavans was confirmed by TLC plate bioassay with C. herbarum and sporeling bioassay with B. cinerea.

Phytoalexin 8 also gave a positive reaction (yellow) with DpNA and the UV absorption spectrum (Fig. 3.7) was similar to the identified hydroxyflavans, with λmax at 208 and 290 nm. This compound was not characterised further.

(ii) Non-phenolic phytoalexins. Purified PA1-4 did not give a positive reaction to TLC plate spray tests with DpNA, vanillin/H_2SO_4 or $NH_2OH.HCL/FeCl_3$ (Table 3.10). They did not quench UV absorbance at 254nm and were visible at 366nm as only faintly fluorescing bands. They did not develop into coloured spots on TLC plates but were visible as water-repellent bands or after charring at 120°C.

The UV absorption spectra of PA1, PA2 and PA3 in MeOH and PA1 in hexane, are illustrated in Figs. 3.8-3.11. In

TABLE 3.10 Visualisation of phytoalexins 1, 2, 3 and 4 on TLC plates

Test[a]		Reaction[b] PA1	PA2	PA3	PA4
UV quenching (254nm)		-	-	-	-
UV fluorescence (366nm)	- alone	VLP	VLP	VLP	VLP
	+ NH_3	VLP	VLP	VLP	VLP
	+ Ethanolic $AlCl_3$	VLP	VLP	VLP	VLP
DpNA	- alone	-	-	-	-
	+ Na_2CO_3	-	-	-	-
Vanillin/H_2SO_4	- immediate	-	-	-	-
	- heated (120°C)	G	DG	G	G
$NH_2OH.HCl/FeCl_3$		-	-	-	-
Water repellency		+	+	+	+
Colour development after 6 weeks		-	-	-	-

a See Methods, section IV, for details.

b -, not visible; VLP, very light purple; G, grey; DG, dark grey; +, water repellent.

MeOH PA1 exhibited maxima at 208, 224 sh and 276 nm, differing only slightly from PA2 (λmax 210, 222 sh and 270 sh nm) and PA3 (λmax 210, 227 sh and 276 sh nm). In hexane the three phytoalexins had peaks c. 236nm with several shoulders down to c. 270nm (Fig 3.11).

Mass spectra of PA1, PA2 and PA3 gave the following information.

PA1: M^+ 169 (34%) (HRMS found M^+ 169.1590, $C_{11}H_{21}O$ requires 169.1592, $C_9H_{19}N_3$ requires 169.1579) with prominent fragment ions at m/e (relative intesnity): 41 (46), 43 (49), 45 (56), 55 (79), 57 (50), 59 (65), 67 (38), 69 (67), 81 (55), 83 (64), 95 (100), 97 (36), 109 (38) and 151 (41).

PA2: M^+ 199 (11%) (HRMS found M^+ 199.1687, $C_{12}H_{23}O_2$ requires 199.1698, $C_{10}H_{21}N_3O$ requires 199.1685) with prominent fragment ions at m/e (relative intesnity) 41 (46), 43 (100), 45 (28), 55 (68), 57 (64), 58 (59), 69 (56), 71 (87), 72 (43), 82 (28), 83 (43), 85 (35), 86 (31), 97 (29) and 185 (36).

PA3: M^+ 213 (27%) (HRMS found M^+ 213.1828, $C_{13}H_{25}O_2$ requires 213.1854, $C_{11}H_{23}N_3O$ requires 213.1841) with prominent fragment ions at m/e (relative intesnity) 29 (27), 41 (63), 43 (100), 45 (28), 55 (70), 57 (50), 58 (67), 59 (27), 69 (43), 71 (70), 83 (35), 85 (30), 86 (28) and 97 (27).

It is particularly interesting that the mass spectra indicate that these phytoalexins may contain nitrogen.

4. Accumulation of phytoalexins

Time course studies were made to investigate changes in phytoalexin concentrations in narcissus bulb scales during

the development of limited and spreading lesions.

Accumulation of total antifungal activity in Et_2O extracts of infected tissue was monitored by B. cinerea sporeling assay and phytoalexins were visualised in chromatograms bioassayed with C. herbarum. Antifungal bands on TLC plates were ascribed to individual phytoalexins according to R_F, colour and water repellency (Table 3.8). The identity of phytoalexins in chromatograms of some extracts was confirmed by co-chromatography with the purified compounds (e.g. for 7-hydroxyflavan in Plate 3.13). However, as complete resolution of phytoalexins was not achieved by a single TLC step it was impossible to distinguish PA2 from PA6, PA3 from PA7 or PA4 from PA8, as the causes of zones of inhibition. Thus, although the phytoalexins probably responsible for antifungal zones are indicated, the accumulation of individual phytoalexins in TLC examinations of extracts is not reported in the text. The accumulation of hydroxyflavan phytoalexins was also measured using HPLC with UV detection.

A. Following inoculation with conidia

The epidermis was removed from bulb scales prior to inoculation to facilitate collection of discrete areas. The symptoms resulting on stripped and unstripped scales following inoculation with B. cinerea or B. narcissicola conidia were similar although the rate of lesion development was more rapid on stripped scales; limited lesions had developed at most sites by the second day after inoculation.

Stripped scales were inoculated with SDW or B. cinerea conidia and incubated in the usual way. At daily intervals

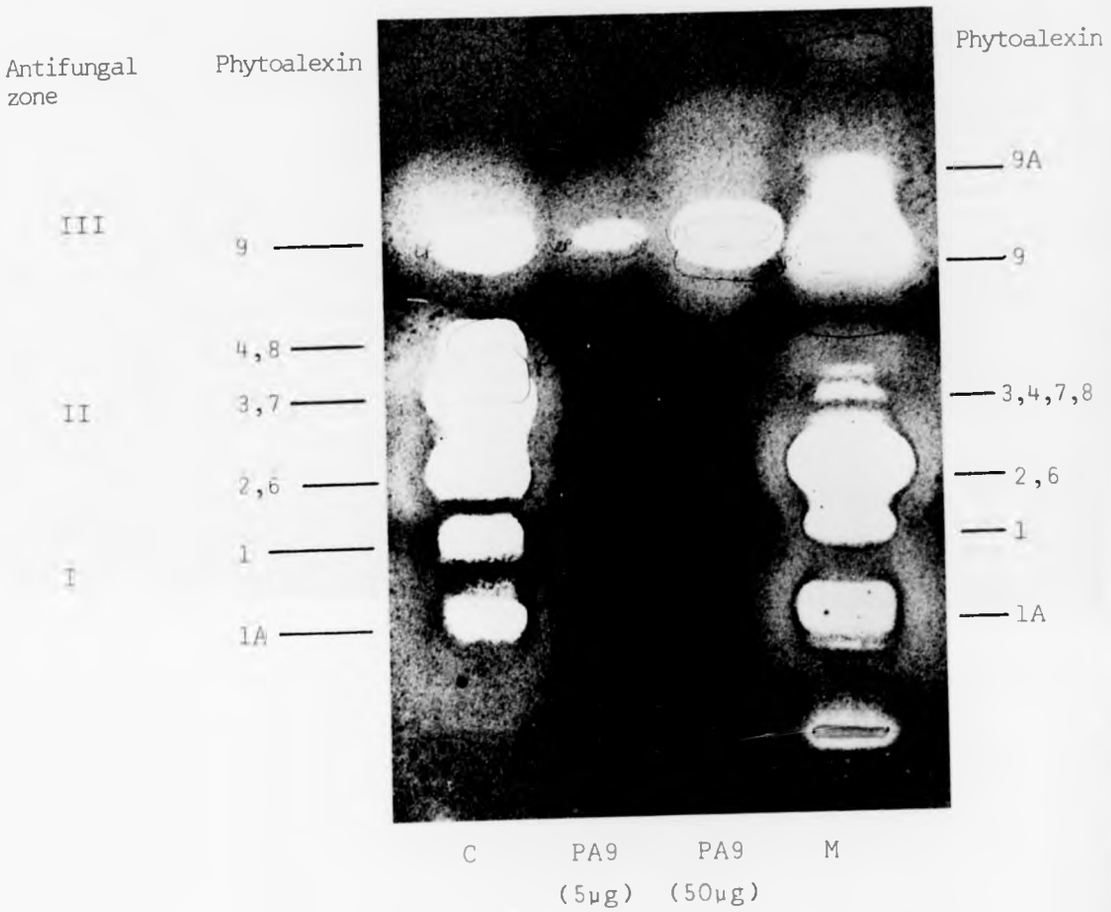
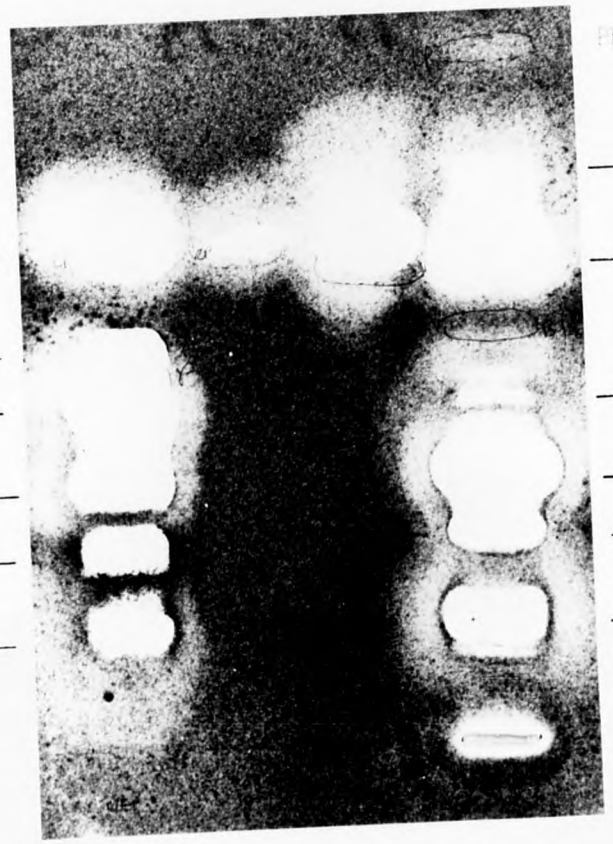


PLATE 3.13 TLC plate bioassay of extracts from bulb scales (0.2g fr.wt.) collected 5 days after inoculation with conidia (C) or mycelium (M) of B. cinerea. Synthetic 7-hydroxyflavan (PA9) was run as a reference compound. The chromatogram was developed in Et_2O-petrol (2:1).


Antifungal zone
Phytoalexin
III
II
I
Phytoalexin
3A
3
2,6,7,8
2,6
1

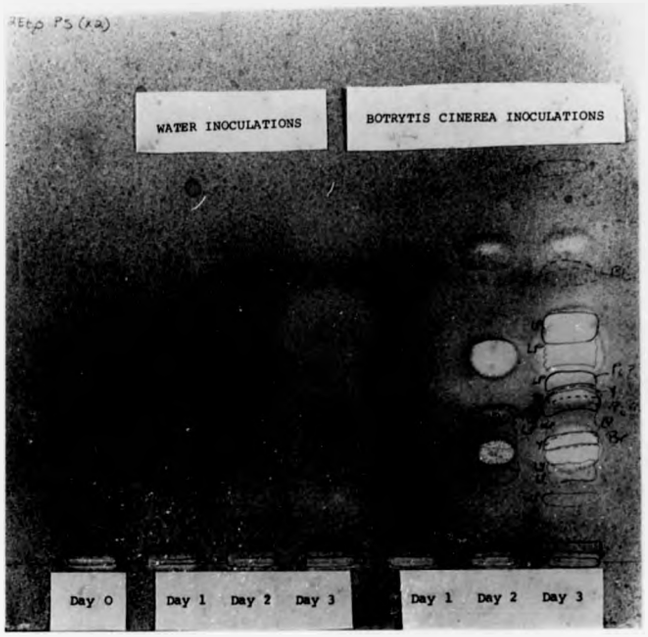
for three days, inoculum droplets (diffusates) and the underlying tissues were separately collected and extracted with Et_2O. The pH of diffusates was measured before extraction. On the third day Et_2O extracted residues were further extracted with MeOH and then AmOH. The antifungal activities of tissue and diffusate extracts were examined by TLC plate bioassay.

In Et_2O extracts of tissue developing limited lesions, phytoalexins were first detected two days after inoculation and the zones of inhibition increased by day three. Three major zones of antifungal activity, containing at least six antifungal compounds, were detected (Plate 3.14).

Antifungal activity in extracts of tissue from beneath water droplets and in all diffusates was slight and did not increase with time after inoculation. No antifungal activity was detected in MeOH and AmOH extracts, confirming that all phytoalexins were extracted with Et_2O.

The pH of both water and B. cinerea inoculum droplets fell with time after inoculation, the fall being greater for B. cinerea diffusates (Table 3.11), which were at pH 3.75 after 2 days.

The total antifungal activity of tissue extracts was examined by B. cinerea sporeling bioassay (Fig. 3.13). Inhibition of germ tube growth by the tissue extract from beneath B. cinerea inocula, tested at 0.5g fr.wt./ml, rose from c. 25% to 100% between the first and second day after inoculation; inhibition by extracts of tissue from beneath water droplets remained at c. 20% throughout.



Phytoalexin	Antifungal zone
9	III
4,8	II
3,7	
2,6	
1	I

PLATE 3.14 TLC plate bioassay of extracts from bulb tissue (0.2g fr.wt.) collected 1,2 and 3 days after inoculation with either SDW or a conidial suspension of B. cinerea. The chromatogram was developed twice in Et_2O-petrol (2:1); PA 1-4, unidentified, non-phenolic phytoalexins; PA6-9, phenolic phytoalexins.

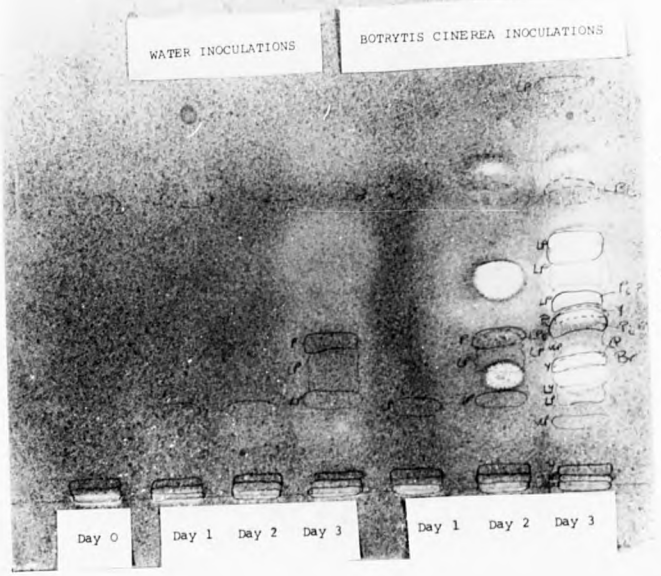

WATER INOCULATIONS
BOTRYTIS CINEREA INOCULATIONS
Day 0
Day 1
Day 2
Day 3
Day 1
Day 2
Day 3

TABLE 3.11 pH of inoculum droplets on bulb scales

Inoculum	pH at daily intervals following inoculation			
	0	1	2	3
Water	6.8	5.10	4.40	4.80
B. cinerea conidia	6.8	4.65	3.75	3.75

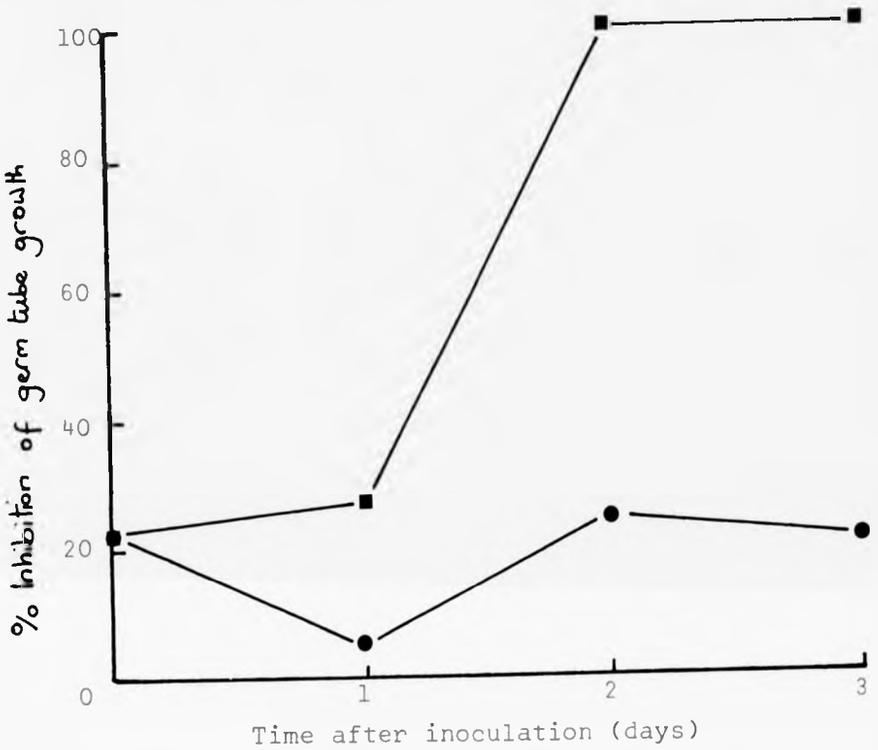


FIG. 3.13 Accumulation of antifungal activity in bulb tissue following inoculation with B. cinerea. Tissue was collected at daily intervals following inoculation with conidia (10^5 spores/ml) of B. cinerea (■—■) or SDW (●—●). The Et_2O extract of tissues was taken to dryness, resuspended in SPN (0.5g fr.wt./ml) and assayed against B. cinerea sporelings.

A quantitative estimate of activity in the three different antifungal zones revealed in TLC plate bioassay (Plate 3.14) was made by B. cinerea sporeling bioassay. An Et_2O extract of limited lesions caused by B. cinerea, collected 3 days after inoculation, was fractionated by PC. The extract from 6g fr.wt. of tissue in 1.5ml MeOH was streaked over two analytical plates (36cm origin) and developed several times in Et_2O-petrol (2:1). Antifungal zones were located from knowledge of their position relative to characteristic UV quenching bands. All fractions were scraped from the plates, eluted in Et_2O and MeOH and, after evaporation to dryness, eluates were resuspended in SPN (0.5 and 0.125g fr.wt./ml).

The antifungal activities of different fractions as revealed by the TLC plate assay (Plate 3.14) was confirmed by B. cinerea sporeling assay (Table 3.12). At the higher concentration tested, considerable activity (50-60% inhibition) was also found in some fractions not expected to be antifungal. This apparently contradictory result is probably a reflection of the problem of locating with precision the limits of antifungal zones on TLC plates not bioassayed.

A comparison of phytoalexin accumulation in bulb scales inoculated with conidia of either B. cinerea or B. narcissicola was made; this experiment also compared phytoalexin production in bulb scales inoculated directly or after removal of the epidermis.

Botrytis cinerea and B. narcissicola conidia caused similar patterns of phytoalexin accumulation in terms of both the number and intensity of antifungal zones appearing

TABLE 3.12 Antifungal activity of phytoalexins partially separated by PC[a]

Fraction	R_F on TLC plate	Antifungal zone [b]	Inhibition of *C. herbarum* [c]	% inhibition of *B. cinerea* germ tube growth[d] 0.5	0.125g fr.wt./ml
1	0 - 0.23		-	54	10
2	0.23 - 0.32	1	+++	75	26
3	0.32 - 0.43		-	58	9
4	0.43 - 0.61	2	+++	100	52
5	0.61 - 0.70		-	60	29
6	0.70 - 0.78	3	+	37	44
7	0.78 - 1.00		-	7	0

a The extract from 5g ft.wt. of infected itssue was applied to analytical TLC plates and chromatograms were developed in Et_2O-petrol (2:1) x 3.

b See Plate 3.14.

c -, no inhibition; +, slight inhibition; +++, strong inhibition.

d Mean of three replicate drops.

in TLC bioassays (Plate 3.15). No antifungal bands were present in the control extract of intact bulb scales. In tissues inoculated after removal of the epidermis one strongly antifungal band (PA3 or PA7) and three weakly antifungal bands were present in the control extracts. Areas of corresponding antifungal zones were greater in extracts from bulb scales inoculated directly; one band (PA4 or PA8) was detected only in tissue inoculated after removal of the epidermis.

B. Following inoculation with mycelium

Bulb scales were inoculated directly with B. narcissicola or B. cinerea mycelium or discs of agar alone (control). One, 2, 3 and 5 days after inoculation, tissue from beneath inocula was collected and extracted with Et_2O and MeOH. Spreading lesions caused by B. narcissicola were either collected as for limited lesions or were collected in two portions, an inner zone of dark brown tissue and an outer circumference, c. 2mm wide, of white, fleshy tissue. Extracts were examined for antifungal activity by TLC plate bioassay (0.4g fr.wt.) and B. cinerea sporeling bioassay (0.1 and 0.01g fr.wt./ml).

The appearance of inhibition zones in TLC plate bioassays (Plate 3.16) closely paralleled the development of limited lesions in bulb tissue inoculated with B. cinerea (Table 3.13). Inhibition zones were first detected two days after inoculation and increased in size and number with time.

Weakly inhibitory zones were present in TLC plate assays of B. narcissicola inoculated tissue two days after inoculation, but these did not increase in number or intensity (Plate 3.16).

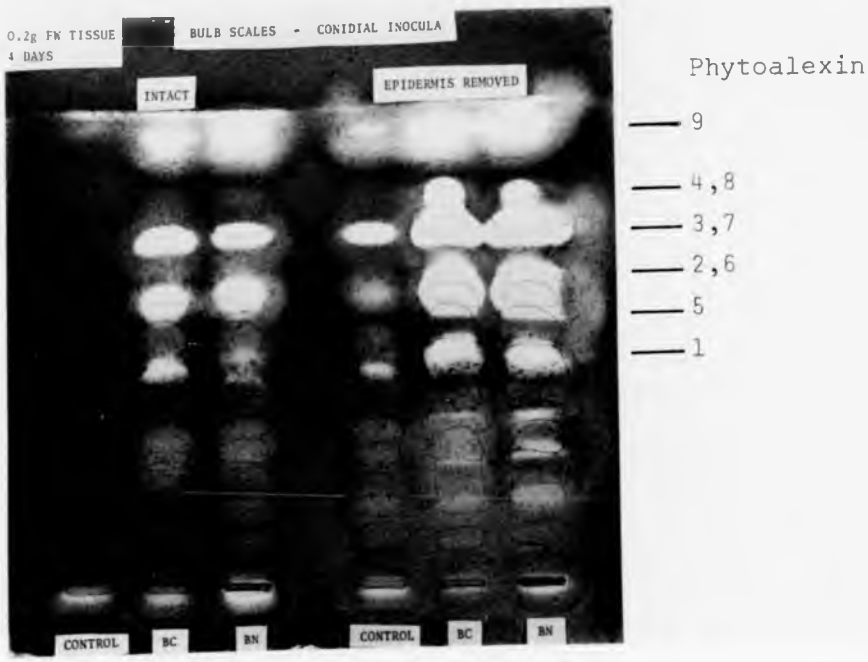
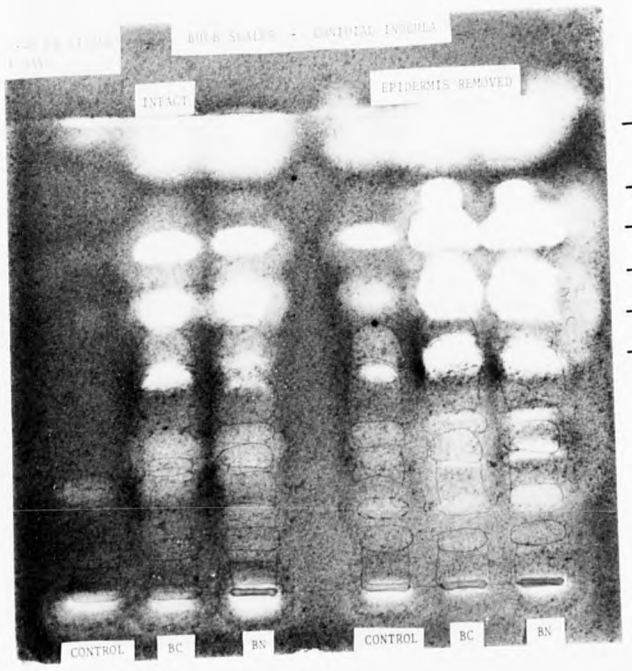


PLATE 3.15 TLC plate bioassay of extracts from bulb scales (0.2g fr.wt.) collected 4 days after inoculation with either SDW (control) or a conidial suspension of B. cinerea (BC), or B. narcissicola (BN). Tissues were inoculated directly or after removal of the epidermis. The chromatogram was developed twice in Et_2O-petrol (2:1); PA1-4, unidentified, non-phenolic phytoalexins; PA6, 7 and 9, hydroxyflavan phytoalexins; PA5 and 8, unidentified phytoalexins, probably phenolic.



Phytoalexin

9

4,8

3,7

2,6

5

1

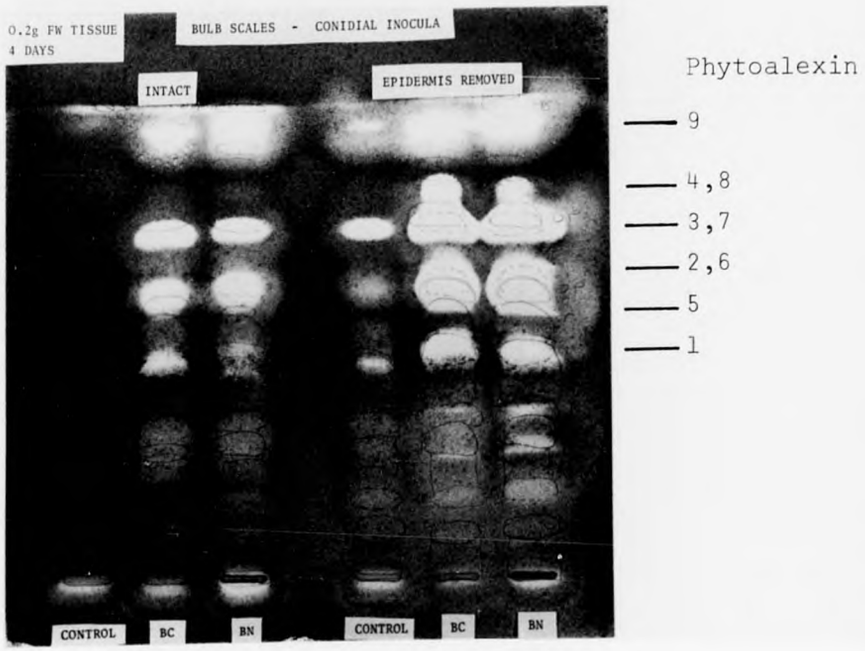
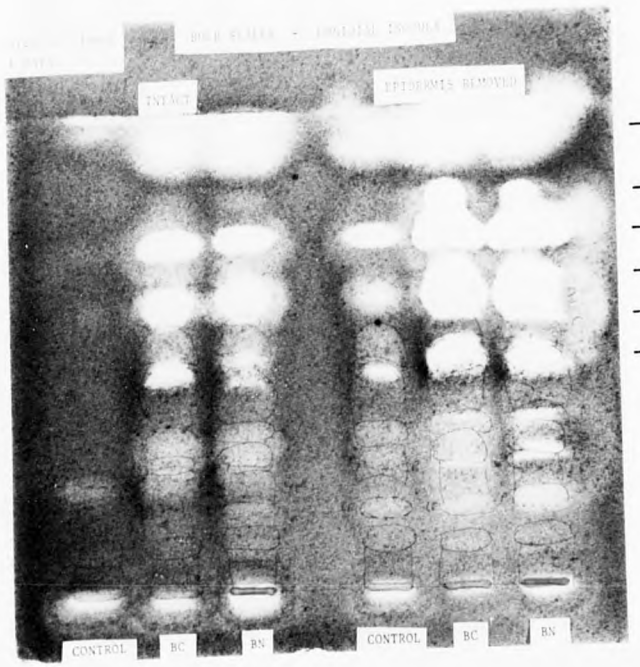


PLATE 3.15 TLC plate bioassay of extracts from bulb scales (0.2g fr.wt.) collected 4 days after inoculation with either SDW (control) or a conidial suspension of B. cinerea (BC), or B. narcissicola (BN). Tissues were inoculated directly or after removal of the epidermis. The chromatogram was developed twice in Et_2O-petrol (2:1); PA1-4, unidentified, non-phenolic phytoalexins; PA6, 7 and 9, hydroxyflavan phytoalexins; PA5 and 8, unidentified phytoalexins, probably phenolic.


INTACT
EPIDERMIS REMOVED
CONTROL
BC
BN
CONTROL
BC
BN

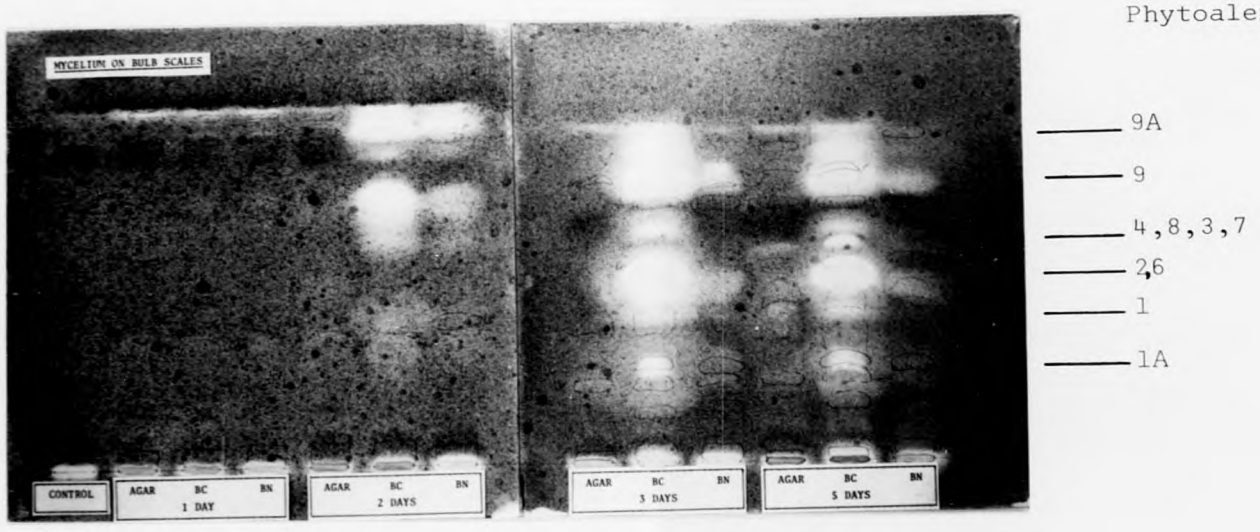
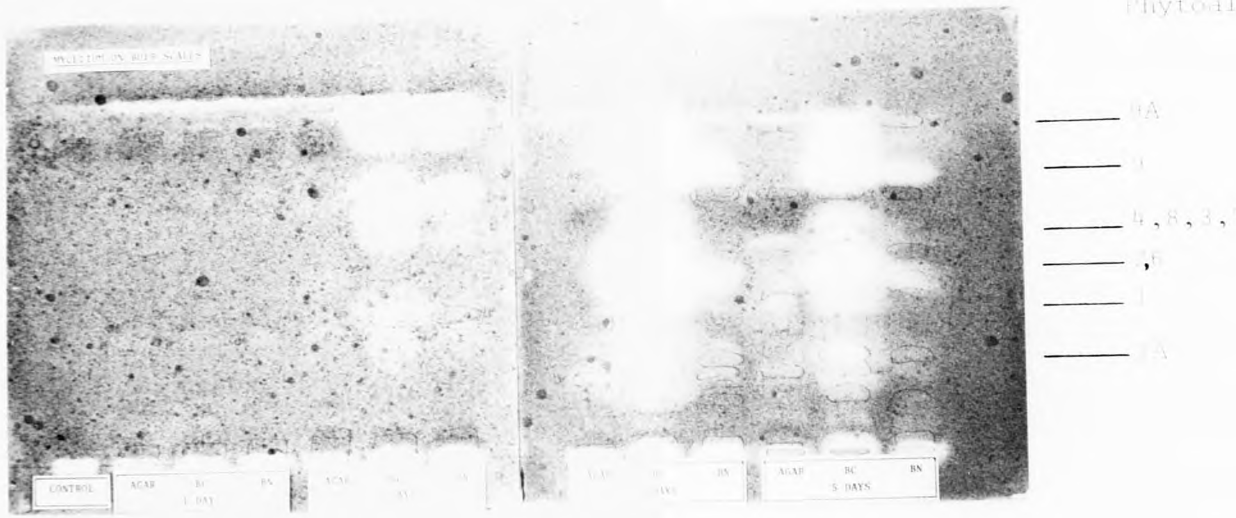


PLATE 3.16 TLC plate bioassay of extracts from bulb tissue (0.4g fr.wt.) collected 1, 2, 3 and 5 days after inoculation with V8 juice agar (control) or mycelium of B. cinerea (BC) or B. narcissicola (BN). The chromatograms were developed twice in Et_2O-petrol (2:1). Phytoalexins as in Plate 3.15.



[illegible] bulb tissue (0.4g fr.wt.) collected [illegible] with [illegible] agar (control) or mycelium of B. cinerea [illegible] were developed twice in Et_2O-petrol (2:1). [illegible]

TABLE 3.13 Lesion development in bulb scale tissues collected for extraction following inoculation with mycelium of B. cinerea or B. narcissicola

Lesion category	% inoculations in each category at intervals (days) after inoculation							
	B. cinerea				B. narcissicola			
	1	2	3	5	1	2	3	5
No lesion	80	45	39	37	30	13	0	0
Limited lesion	20	51	53	57	65	13	1	0
Spreading lesion	0	4	8	6	5	74	99	100

When spreading lesions caused by B. narcissicola were collected in two portions, TLC plate assays again revealed only weakly inhibitory bands, mainly from tissue at the edge of spreading lesions.

The different patterns of phytoalexin accumulation revealed by TLC plate bioassay were also evident, though less pronounced, in bioassays against sporelings of B. cinerea (Table 3.14).

Four species of Botrytis, B. cinerea, B. fabae, B. narcissicola and B. tulipae, were compared for their ability to elicit phytoalexins in bulb tissue. Cladosporium herbarum was inhibited strongly only in the chromatogram of the extract of B. cinerea inoculated tissue (Plate 3.17); this was also the only species to cause large numbers of limited lesions. Weakly antifungal zones were visible in extracts of tissue from beneath B. tulipae and B. narcissicola inocula (causing some limited lesions and spreading lesions respectively) but not from beneath B. fabae or agar inocula (causing no visible symptoms).

C. Measurement of hydroxyflavan phytoalexin accumulation by HPLC

The usefulness of HPLC as a tool for measuring hydroxyflavan phytoalexin levels was indicated by the one-step fractionation of crude extract (Section 3B(ii)). Conditions required for rapid separation of 7-hydroxyflavan (PA9), 7,4'-dihydroxyflavan (PA6) and 7,4'-dihydroxy-8-methylflavan (PA7) were investigated.

Isocratic elution with 35% MeOH in 5% HCO_2H (eluent A) resolved 34 peaks in c. 70 mins (Fig. 3.14). The positions of PA6, PA7, PA8 and PA9 were determined by co-injection with

TABLE 3.14 Antifungal activity of extracts of bulb scale tissues following inoculation with mycelium of B. cinerea or B. narcissicola

Inoculum[a]	% inhibition of B. cinerea germ tube growth at intervals (days) after inoculation[b]									
	0.1g fr.wt./ml					0.01g fr.wt./ml				
	0	1	2	3	5	0	1	2	3	5
Control (agar)	11	35	39	30	38	0	0	0	6	4
B. cinerea	-[c]	62	85	92	86	-	0	26	33	30
B. narcissicola (inner)	-	-	47	34	50	-	-	0	0	0
B. narcissicola (outer)	-	46	35	30	32	-	7	0	11	18

a Tissue collected from beneath inocula was extracted with Et_2O. Spreading lesions caused by B. narcissicola were collected in two portions, an inner zone of dark brown tissue and an outer circumference (c. 2mm wide) of white tissue.

b Extracts were assayed against B. cinerea sporelings in SPN.

c Not determined.

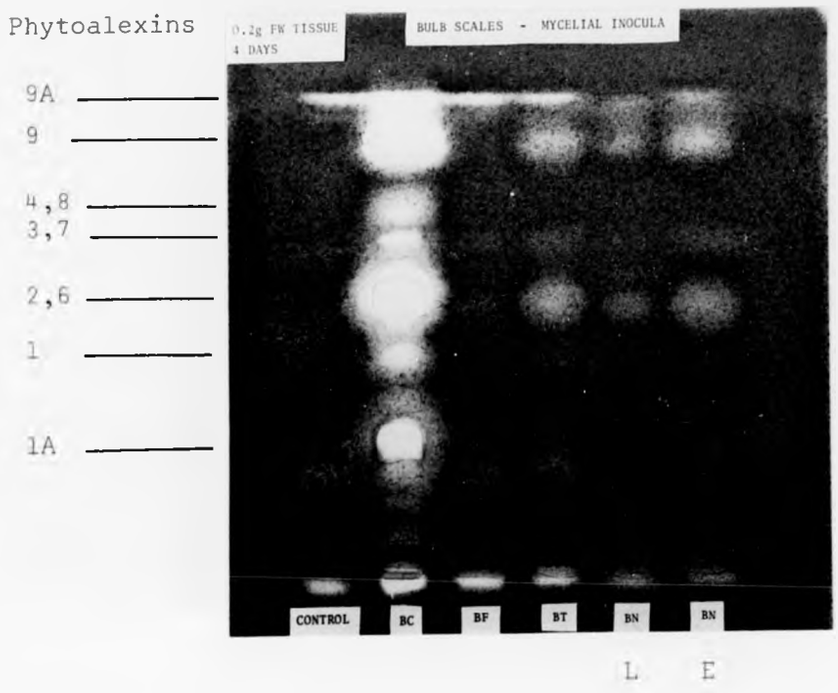
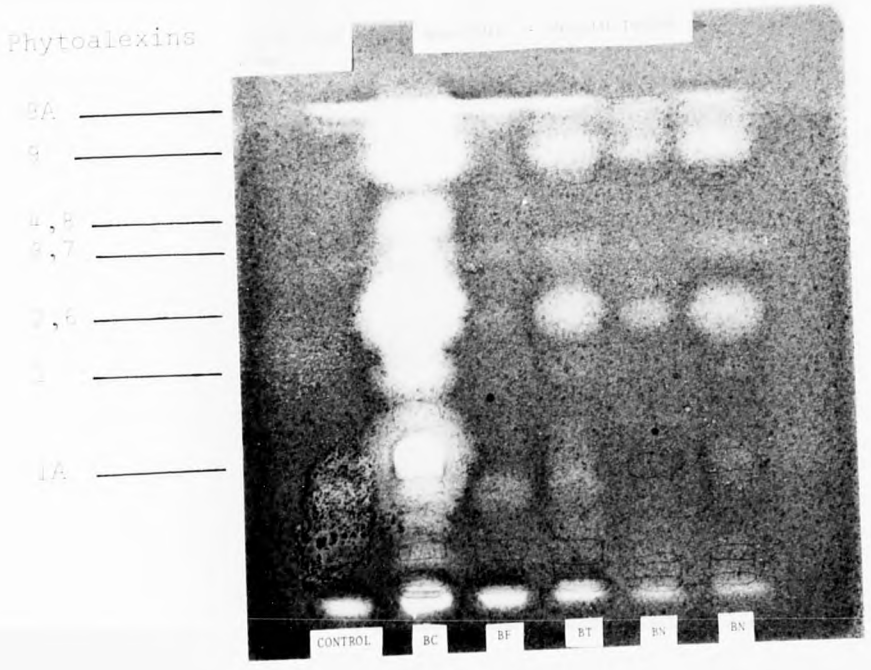


PLATE 3.17 TLC plate bioassay of extracts from bulb scales (0.4g fr.wt.) prepared 4 days after inoculation with V8 juice agar (control) or mycelial inocula of *B. cinerea* (BC), *B. fabae* (BF), *B. tulipae* (BT) or *B. narcissicola* (BN). Spreading lesions caused by *B. narcissicola* were collected in two portions, the lesion itself (L) and a band of tissue, c. 2mm wide, from the edge (E). The chromatogram was developed twice in Et_2O-petrol (2:1).


Phytoalexins
9A
9
4,8
3,7
2,6
5
1A
CONTROL
BC
BF
BT
BN
BN

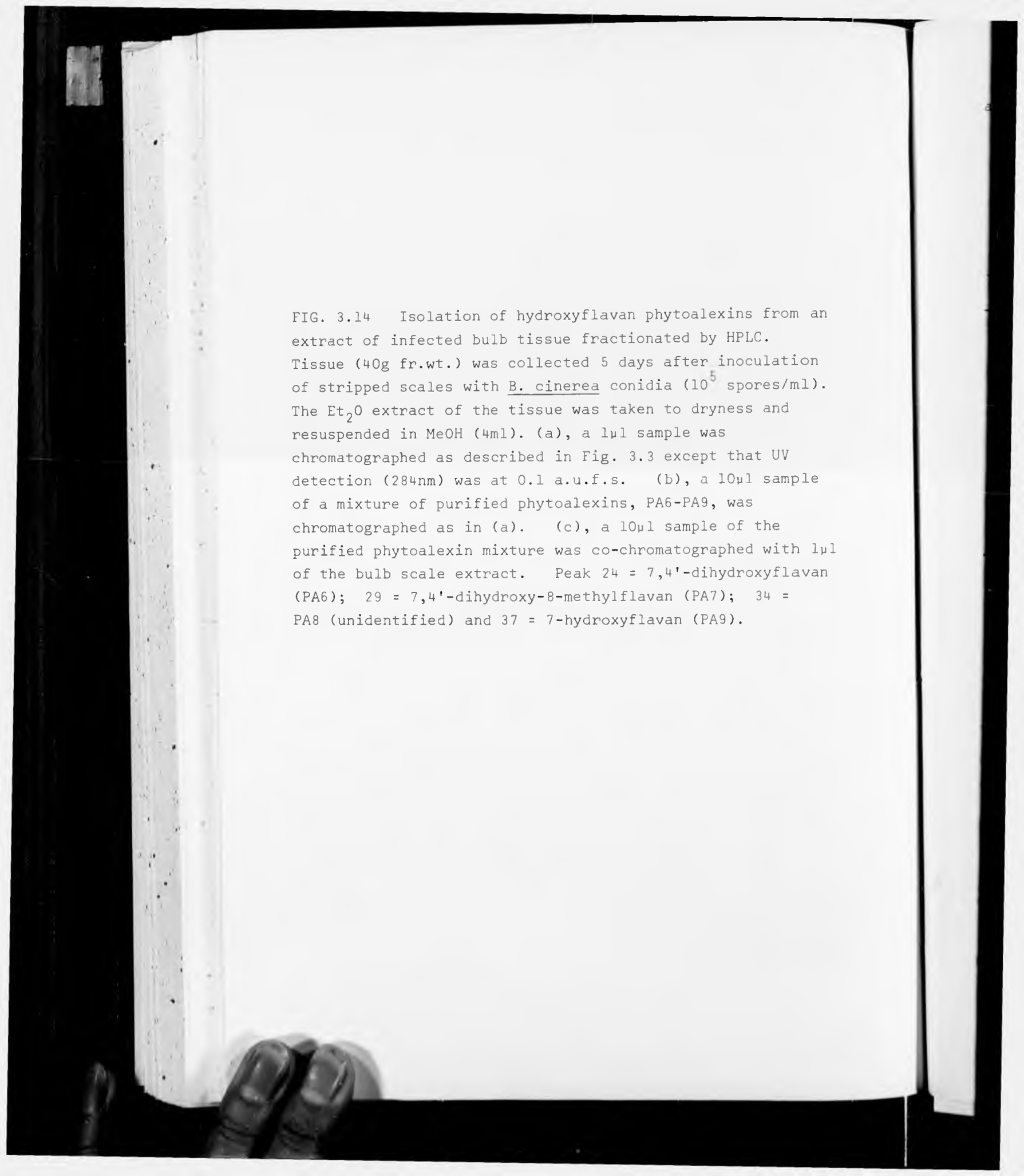


FIG. 3.14 Isolation of hydroxyflavan phytoalexins from an extract of infected bulb tissue fractionated by HPLC. Tissue (40g fr.wt.) was collected 5 days after inoculation of stripped scales with B. cinerea conidia (10^5 spores/ml). The Et_2O extract of the tissue was taken to dryness and resuspended in MeOH (4ml). (a), a 1µl sample was chromatographed as described in Fig. 3.3 except that UV detection (284nm) was at 0.1 a.u.f.s. (b), a 10µl sample of a mixture of purified phytoalexins, PA6-PA9, was chromatographed as in (a). (c), a 10µl sample of the purified phytoalexin mixture was co-chromatographed with 1µl of the bulb scale extract. Peak 24 = 7,4'-dihydroxyflavan (PA6); 29 = 7,4'-dihydroxy-8-methylflavan (PA7); 34 = PA8 (unidentified) and 37 = 7-hydroxyflavan (PA9).

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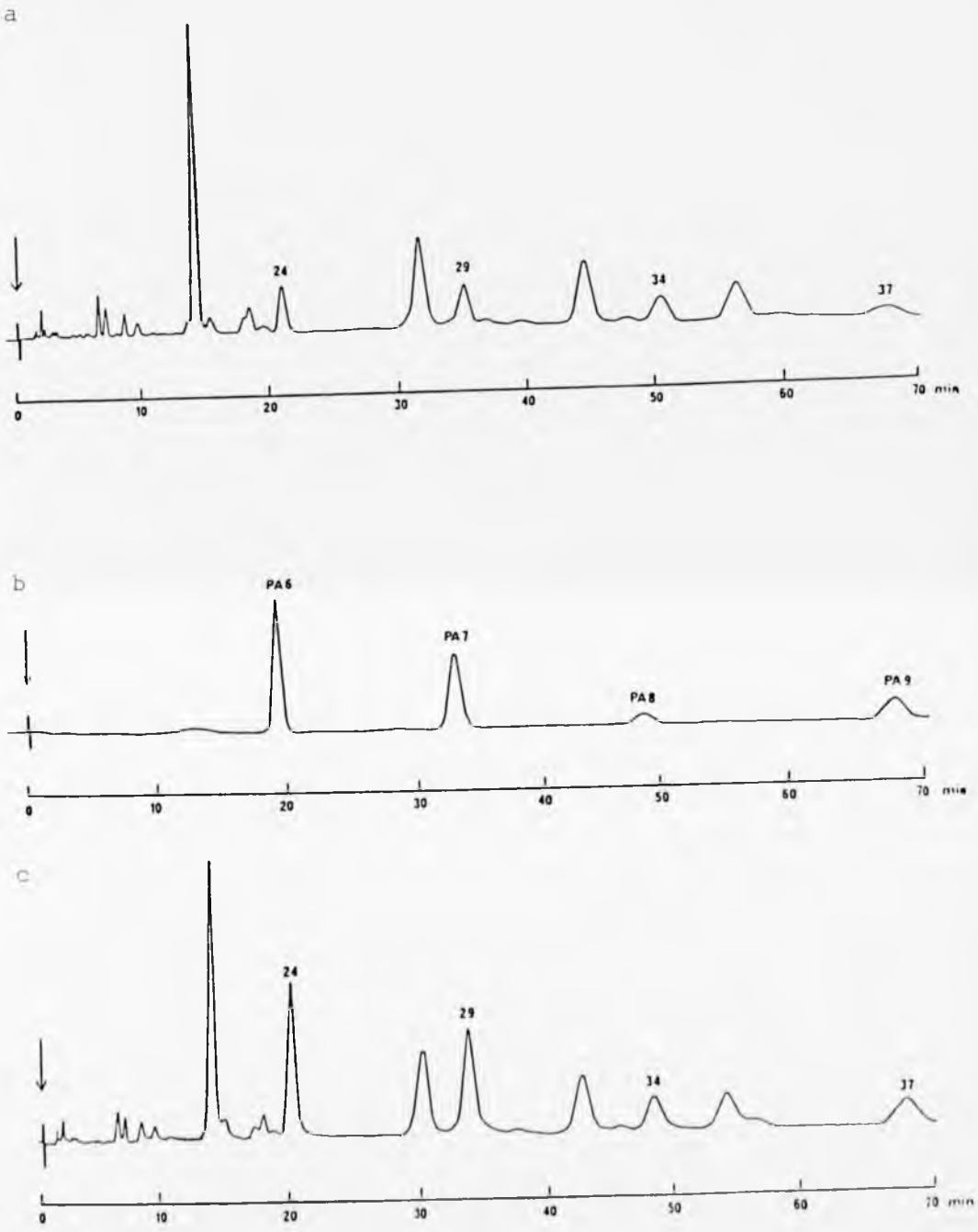

FIG. 3.14

a mixture of the purified phytoalexins (Table 3.15 and Fig. 3.14). Retention times were 21.2 (PA6), 35.2 (PA7), 50.7 (PA8) and 69.6 (PA9) mins.

Isocratic elution with MeOH-MeCN-HCO_2H (40:10:50), eluent B, reduced the 'tailing' of later eluting peaks and the total elution time to c. 20 mins; however, interference between neighbouring peaks was considerable.

The application of gradient elution conditions, running linearly over 30 mins from MeOH-5% HCO_2H (35:65) to MeOH-MeCN-HCO_2H (40:10:50), from 5% to 100% eluent B, effected good resolution of most components in only c. 30 mins (Fig. 3.15a). Curvilinear elution (programme 7) increased retention times slightly but reduced the interference of neighbouring peaks with PA6 (Fig. 3.15b). This programme was therefore used in a semi-quantitative investigation of the accumulation of hydroxyflavan phytoalexins in tissues undergoing a resistant response (Table 3.16).

Extracts of tissue collected after inoculation of bulb scales with B. cinerea mycelium, previously analysed by TLC (section 4B), were re-analysed by HPLC. Samples equivalent to the extract from 40mg fr.wt. of infected tissue were injected in 10 µl MeOH. The compounds were not detected in extracts of agar inoculated tissue. 7,4'-Dihydroxyflavan (PA6) was detected one day after inoculation with B. cinerea and was present at higher levels on subsequent days. Five days after inoculation the four purified hydroxyflavan phytoalexins (PA6-9) were all detected.

It must be emphasised, however, that because of the different UV absorptions of PA6-9 at 284nm, the results

TABLE 3.15 Location of phytoalexins in HPLC chromatograms by 'spiking' with a mixture of the purified compounds[a]

Phytoalexin	Peak Number	Peak area (mm^2) Extract alone	Purified Phytoalexins	Extract mixed with phytoalexins Observed	Calculated
PA6	24	57.8	132.0	191.8	189.8
PA7	29	66.5	126.0	185.3	192.5
PA8	34	52.0	18.0	75.0	70.0
PA9	37	38.0	49.0	80.8	79.0

a Chromatogram traces and further details are given in Fig. 3.14.

FIG. 3.15 Separation of hydroxyflavan phytoalexins from an extract of infected bulb tissue by HPLC using gradient elution. An extract was prepared as described in Fig. 3.3. (a), 1µl of extract chromatographed by gradient elution at 5ml/min. Initial solvent conditions were MeOH - 5% HCO_2H (35:65), running linearly over 30 mins to final conditions MeOH - MeCN - 5% HCO_2H (40:10:50). UV detection was at 284nm, 0.1 a.u.f.s. (b), 1µl of extract chromatographed with curvilinear gradient elution (Waters solvent programmer, curve 7). (c), mixture of purified phytoalexins (5µl) chromatographed as in (a). Phytoalexin peaks (24, 29, 34, 37) as in Fig. 3.3.

an phytoalexins from
HPLC using gradient
described in Fig. 3.3.
gradient elution at
were MeOH - 5%
30 mins to final
:10:50). UV
(b), lµl of extract
ient elution (Waters
mixture of purified
in (a). Phytoalexin
.

FIG. 3.15a

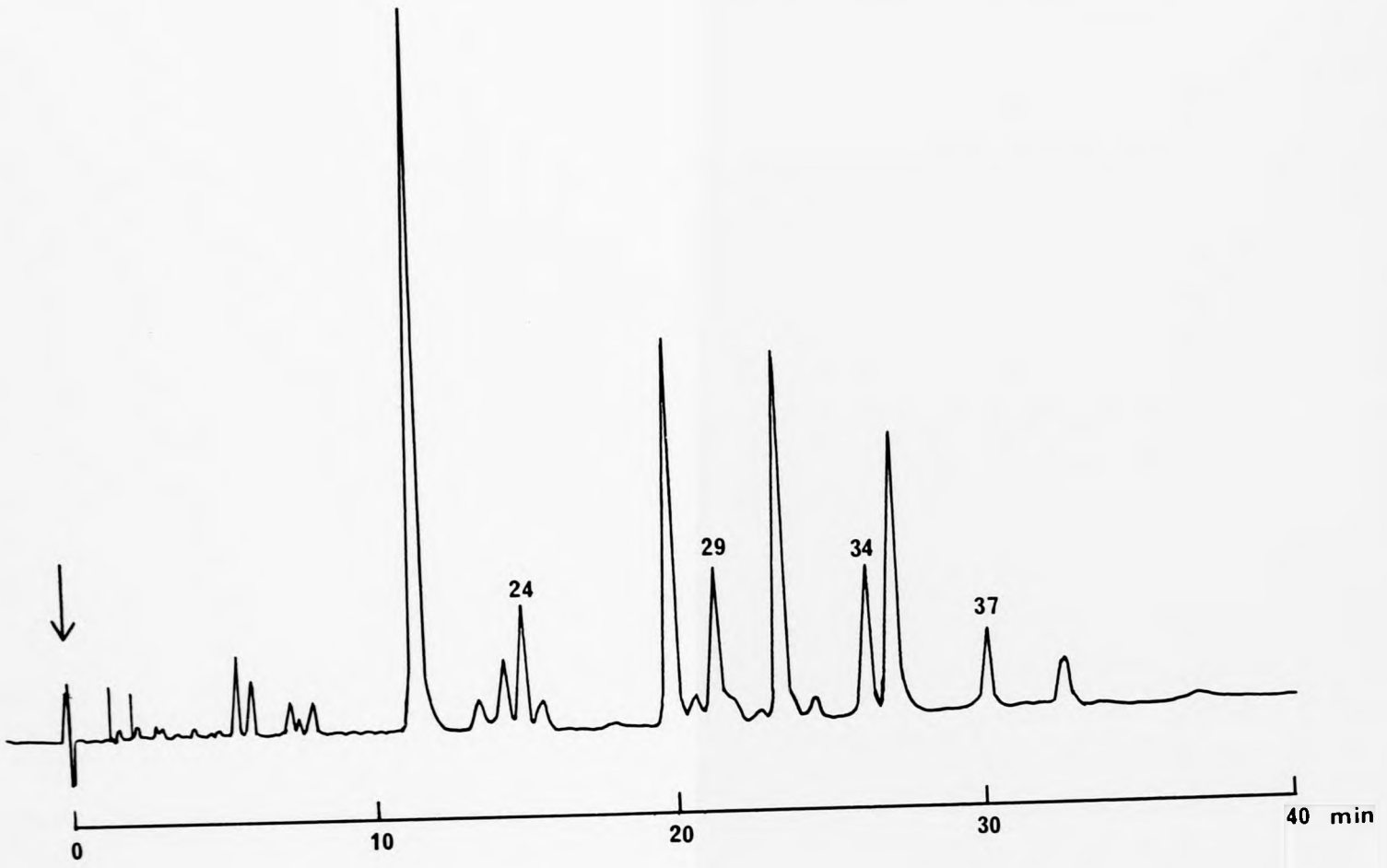

FIG. 3.15b

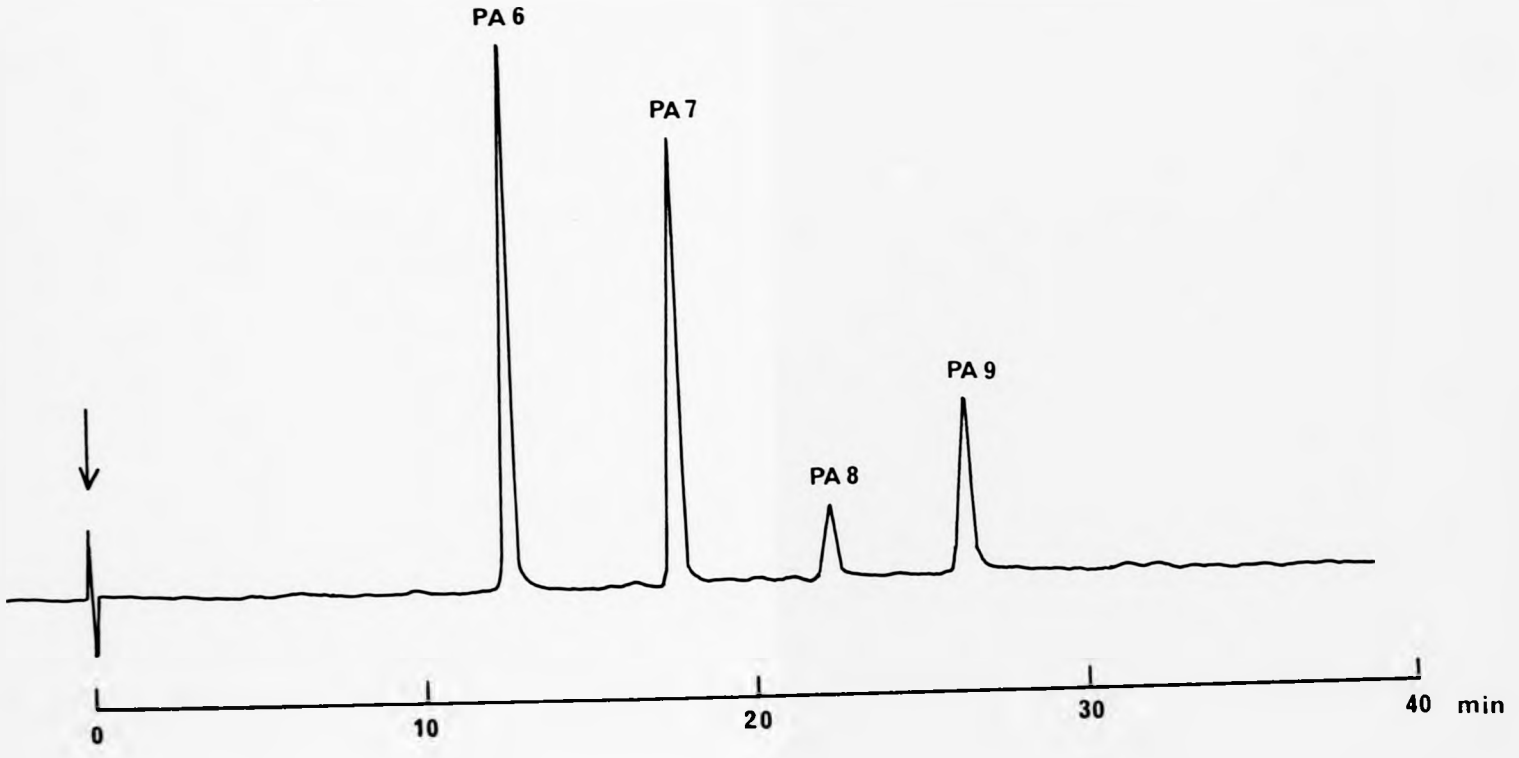

FIG. 3.15c

TABLE 3.16 Measurement of hydroxyflavan phytoalexin accumulation by UV absorbance (284nm) in bulb scale extracts analysed by HPLC[a]

Time after inoculation (days)	Phytoalexin peak areas (mm^2) in tissue extracts[b] Control inocula PA6	PA7	PA8	PA9	Number of peaks	*B. cinerea* inocula PA6	PA7	PA8	PA9	Number of peaks
0	0	0	0	0	7	0	0	0	0	7
1	0	0	0	0	15	< 1	0	0	0	20
2	0	0	0	0	27	9	< 1	< 1	< 1	28
3	-[c]	-	-	-	-	36	< 1	0	< 1	23
5	0	0	0	0	36	76	43	15	4	31

a See text for details elution.

b Extracts of 40mg fr.wt. of inoculated tissue were injected in 10µl MeOH. Peak areas were measured at 284nm, λmax of PA6 (7,4'-dihydroxyflavan) from the equation: area = peak width at base x peak height x 0.5. Detection was at 0.1 absorbance units full scale deflection (a.u.f.s.).

c Not determined.

obtained are only semi-quantitative; calibration with known amounts of purified phytoalexins is required. A rigorous quantitative investigation also demands repeated analyses and the use of an internal standard.

5. Fungitoxicity of bulb scale phytoalexins

The fungitoxicity of the purified phytoalexins and crude bulb scale extracts were tested against B. cinerea, B. fabae, B. narcissicola and B. tulipae. Fungitoxicity was assessed by sporeling bioassay in SPN and Czapek Dox, and fungicidal activity by vital staining with trypan blue. Purified phytoalexins were tested at a range of concentrations and ED_{50} values calculated.

A. Crude (unfractionated) extract tested against four Botrytis species

A crude Et_2O extract of bulb scale tissue bearing limited lesions, collected three days after inoculation with B. cinerea conidia, was assayed against sporelings at concentrations ranging from 0.005 to 0.05g fr.wt./ml. Sporeling viability was assessed after 18h exposure to crude extract in SPN, at 0.1g fr.wt./ml.

The four species were inhibited to a similar extent in the sporeling growth assay, all sporelings being inhibited by $\leq$10% at 0.0125g fr.wt./ml and >70% at 0.025g fr.wt./ml (Table 3.17). In the viability test it was again found that B. narcissicola was no more tolerant to narcissus phytoalexins than the other Botrytis species. More than 90% of B. cinerea, B. narcissicola and B. tulipae sporelings were killed completely (i.e. all cells) after 18h exposure to 0.1g fr.wt./ml; B. fabae was slightly more

TABLE 3.17 Inhibition of germ tube growth of four Botrytis species by an ether extract of bulb tissue bearing limited lesions[a]

Species	Extract conc. (g fr.wt./ml)	Germ tube[b] length (µm) at 24h	% inhibition
	0	509 ± 26	-
	0.005	554 ± 31	0
B. cinerea	0.0125	546 ± 30	0
	0.025	111 ± 7	84
	0.05	28 ± 2	100
	0	438 ± 21	-
	0.005	>500	0
B. fabae	0.0125	564 ± 21	0
	0.025	147 ± 7	73
	0.05	27 ± 1	100
	0	299 ± 12	-
	0.005	287 ± 11	4
B. narcissicola	0.0125	277 ± 11	8
	0.025	73 ± 2	78
	0.05	11 ± 1	100
	0	458 ± 25	-
	0.005	-	-
B. tulipae	0.0125	415 ± 21	10
	0.025	-	-
	0.05	25 ± 2	100

a Sporelings of each species were assayed against the extract in SPN.

b Mean ± SEM.

tolerant (Table 3.18).

B. Purified phytoalexins tested against four Botrytis species

Phytoalexin 1 (not identified), 7,4'-dihydroxyflavan (PA6) and 7,4'-dihydroxy-8-methylflavan (PA7) were assayed against three isolates of B. narcissicola and one isolate each of B. cinerea, B. fabae and B. tulipae. Bioassays were performed before identification of the compounds and concentrations were therefore adjusted to standard optical densities. From subsequent identification and calculation of UV absorption extinction coefficients it was possible to determine the concentrations at which phytoalexins were tested; these concentrations are therefore given in Tables 3.19 and 3.20.

None of the phytoalexins was found to be less active, either fungitoxic (Table 3.19) or fungicidal (Table 3.20), towards sporelings of B. narcissicola than any of the other three species. Differences between species were not large but, in general, sporeling growth of B. fabae and B. tulipae was inhibited the least (Table 3.19) and spores of B. fabae and B. cinerea were killed the least frequently (Table 3.20).

C. Comparison of purified phytoalexins

The six phytoalexins purified in milligram quantities were assayed against B. cinerea sporelings in SPN. Phytoalexin concentrations were adjusted to a standard optical density in MeOH and a 10 or 100-fold dilution series tested. ED_{50} values were determined from plots of phytoalexin concentration (absorbance units) against % inhibition of germ tube growth and converted to absolute values (µM or µg/ml) from UV absorbance extinction coefficients obtained subsequently.

TABLE 3.18 Killing of sporelings of four Botrytis species by an ether extract of bulb tissue bearing limited lesions[a]

Species	% sporelings in each category[b]		
	All cells dead	Germ tube dead	All cells alive
B. cinerea	93 (89-96)	7 (4-11)	0
B. fabae	72 (67-81)	27 (19-31)	1 (0-2)
B. narcissicola 1	100	0	0
B. narcissicola 2	98 (96-100)	1 (0-4)	1 (0-2)
B. narcissicola 3	98 (95-100)	1 (0-4)	1 (0-2)
B. tulipae	100	0	0

a Sporelings in SPN were exposed to the extract (0.1g fr.wt. of tissue/ml) for 18h and then assessed for viability by staining with trypan blue.

b Mean of three replicates in which at least 100 sporelings were examined; ranges between replicates are given in parentheses.

TABLE 3.19 Inhibition of germ tube growth of four Botrytis species by three bulb tissue phytoalexins[a]

Phytoalexin	Species	Concentration of phytoalexin[b] χ Germ tube length (μm)[c]	Inhibition (%)	χ/2 Germ tube length (μm)[c]	Inhibition
PA1					
	B. cinerea	497 ± 22	41	593 ± 40	28
	B. fabae	379 ± 21	33	563 ± 29	0
	B. narcissicola 1	319 ± 15	53	491 ± 20	23
	B. narcissicola 2	329 ± 15	51	412 ± 17	37
	B. narcissicola 3	351 ± 19	56	493 ± 18	35
	B. tulipae	537 ± 28	18	591 ± 30	9
7,4'-Dihydroxyflavan					
(PA6)	B. cinerea	570 ± 25	31	597 ± 34	27
	B. fabae	421 ± 18	25	565 ± 23	0
	B. narcissicola 1	216 ± 11	71	578 ± 21	9
	B. narcissicola 2	230 ± 21	69	445 ± 17	31
	B. narcissicola 3	470 ± 19	39	564 ± 21	28
	B. tulipae	504 ± 23	23	620 ± 29	4
7,4'-Dihydroxy-8-methylflavan					
(PA7)	B. cinerea	97 ± 6	98	331 ± 31	65
	B. fabae	116 ± 6	83	576 ± 24	0
	B. narcissicola 1	115 ± 12	88	592 ± 27	6
	B. narcissicola 2	127 ± 7	87	290 ± 17	58
	B. narcissicola 3	259 ± 13	70	468 ± 22	29
	B. tulipae	245 ± 11	67	455 ± 27	32

a Sporelings of each species were assayed against the phytoalexins in SPN. Germ tube lengths in SPN alone (control) were: B. cinerea, 789±31μm; B. fabae, 550±33; B. narcissicola 1, 628±23; B. narcissicola 2, 621±28; B. narcissicola 3, 732±35 and B. tulipae 645±26 μm at 24h.

b Hydroxyflavan phytoalexins were assayed at concentrations giving optical densities 1 and 0.5 (χ and χ/2), and PA1 at 0.1 and 0.5, at λmax in MeOH. Concentrations (χ) were subsequently determined from ελmax as: PA1, 633μM; 7,4'-dihydroxyflavan, 214μM and 7,4'-dihydroxy-8-methylflavan, 249μM. Germ tube growth of all species was completely inhibited with each phytoalexin at a concentration 5χ.

c Mean ± SEM.

TABLE 3.20 *Killing of sporelings of four* Botrytis *species by three bulb tissue phytoalexins*[a]

Phytoalexin	Species	% sporelings in each category[b] All cells dead	Germ tube dead	All cells alive
PA1				
	B. cinerea	60 (49-72)	40 (28-51)	0
	B. fabae	78 (65-85)	22 (15-35)	0
	B. narcissicola 1	99 (98-100)	1 (0-2)	0
	B. narcissicola 2	98 (96-100)	1 (0-2)	1 (0-2)
	B. narcissicola 3	97 (95-100)	3 (0-9)	0
	B. tulipae	96 (94-100)	2 (0-6)	2 (0-4)
7,4'-Dihydroxyflavan				
(PA6)	*B. cinerea*	60 (42-68)	36 (32-48)	4 (0-10)
	B. fabae	78 (52-92)	22 (8-48)	0
	B. narcissicola 1	94 (87-100)	6 (0-13)	0
	B. narcissicola 2	89 (86-92)	11 (8-14)	0
	B. narcissicola 3	100	0	0
	B. tulipae	100	0	0
7,4'-Dihydroxy-8-methylflavan				
(PA7)	*B. cinerea*	98 (95-100)	2 (0-5)	0
	B. fabae	82 (57-100)	18 (0-43)	0
	B. narcissicola 1	100	0	0
	B. narcissicola 2	99 (97-100)	1 (0-3)	0
	B. narcissicola 3	98 (92-100)	2 (0-8)	0
	B. tulipae	100	0	0

a Sporelings in SPN were exposed for 18h to PA1 at 3.16mM, 7,4'-dihydroxyflavan at 1.07mM and 7,4'-dihydroxy-8-methylflavan at 1.25mM and then assessed for viability by staining with trypan blue.

b Mean of three replicates in which at least 50 sporelings were examined; ranges between replicates are given in parentheses.

The results (Table 3.21) placed the phytoalexins in the following order of activity:
7-hydroxyflavan (PA9) > 7,4'-dihydroxy-8-methylflavan (PA7) > 7,4'-dihydroxyflavan (PA6) > PA2 > PA1 > PA3.

A comparison of the antifungal activities of the flavonoid phytoalexins against C. herbarum in a TLC plate spot bioassay again revealed 7,4'-dihydroxyflavan as the least active of the three (Table 3.22 and Plate 3.18).

D. Comparison of synthetic and natural phytoalexins

The antifungal activities of synthetic (racemic) and natural phytoalexins were compared by bioassay against B. cinerea sporelings in SPN or Czapek Dox, using the solution addition method. Synthetic and natural 7,4'-dihydroxyflavan and 7,4'-dihydroxy-8-methylflavan were assayed at several concentrations around the ED_{50} values recorded previously for the natural compounds (Table 3.21).

In SPN the ED_{50} of synthetic and natural phytoalexins were similar (Table 3.23) while in Czapek Dox natural compounds appeared to be the more active. The differences in activity were considerably less than two-fold, indicating that optical isomers of the flavonoids possessed similar activity.

The different ED_{50} values obtained for natural 7,4'-dihydroxy-8-methylflavan against B. cinerea sporelings in SPN by the solution addition method (48μM) and the solution replacement method (125μM) illustrates the difficulty of comparing the activity of phytoalexins using even slightly different methods of bioassay.

TABLE 3.21 Inhibition of germ tube growth of B. cinerea by six phytoalexins from narcissus bulb tissue

Phytoalexin	Concentration[a] (absorbance units)	Germ tube length[b] (µm)	Inhibition[c] (%)	ED_{50} (µM)	ED_{50} (µg/ml)
PA1	0.1	302 ± 29	53		
	0.5	80 ± 3	95	734	124.0
	1.0	57 ± 2	99		
PA2	0.1	452 ± 14	25		
	0.5	142 ± 9	83	346	68.8
	1.0	53 ± 2	100		
PA3	0.1	457 ± 27	24		
	0.5	96 ± 5	92	1149	244.8
	1.0	57 ± 2	99		
7,4'-Dihydroxyflavan (PA6)	0.1	478 ± 18	2		
	0.5	313 ± 16	27	266.9	64.6
	1.0	296 ± 20	31		
	5.0	54 ± 2	100		
7,4'-Dihydroxy-8-methylflavan (PA7)	0.1	440 ± 18	11		
	0.5	150 ± 13	65	124.2	31.8
	1.0	112 ± 12	74		
	5.0	54 ± 2	100		
7-Hydroxyflavan (PA9)	0.1	521 ± 2	0		
	1.0	123 ± 6	85	97.7	22.1
	5.0	55 ± 2	100		

a Measured at UV λmax in MeOH.

b Mean ± SEM. In SPN alone (control) B. cinerea sporelings were 488 ± 23µM at 24h.

c ED_{50} in absorbance units converted to µM and µg/ml from subsequent determination of ελmax for each phytoalexin. ελmax only approximate for PA1-PA3.

TABLE 3.22 Inhibition of C. herbarum growth on TLC plates by hydroxyflavan phytoalexins[a]

Phytoalexin	Inhibition of C. herbarum					
	2.5	5	10	25	50	100 nmoles
(±)-7-Hydroxyflavan	0	-	2	-	4	4
(±)-7,4'-Dihydroxyflavan	0	0	2	2/4	3/4	4
(±)-7,4'-Dihydroxy-8-methylflavan	0	1	2	4	4	4
(-)-7,4'-Dihydroxy-8-methylflavan	0	1	-	4	4	-

a Samples (20µl) were spotted onto TLC plates over a constant area (c. 30mm^2).

b 0, no inhibition; 1, slight inhibition; 2, moderate inhibition; 3, inhibition over all the application area; 4, inhibition outside the application area; -, not determined.

i.e.

TABLE 3.23 Comparison of the antifungal activity of natural and synthetic hydroxyflavan phytoalexins by bioassay against sporelings of B. cinerea

Bioassay medium	Phytoalexin	Conc. (µM)	Synthetic compounds GTL[b] (µm)	Synthetic compounds I (%)	Synthetic compounds ED_{50} (µM)	Natural compounds GTL[b] (µm)	Natural compounds I (%)	Natural compounds ED_{50} (µM)
SPN (pH4)	7,4'-Dihydroxy-8-methylflavan (PA7)	50	227±10	30		218±11	33	
		100	161± 7	54	48	150± 7	58	43
		200	48± 2	94		37± 2	98	
	Repeat experiment	50	212± 7	26		250± 9	10	
		100	123± 9	62	42	198±10	46	53
		200	32± 1	98		37±2	98	
Czapek Dox (pH 6.8)	7,4'-Dihdroxy-flavan (PA6)	1	383±16	0		435±12	0	
		10	314±12	20		246±11	40	
		100	273±13	32	170	242±12	41	145
		250	135± 6	72		135± 8	72	
		500	37±1	100		41± 2	100	
	7,4'-Dihydroxy-8-methylflavan (PA7)	1	360±14	6		333±17	14	
		10	222±16	42		223±14	42	
		100	190± 8	59	40	128± 7	74	34
		250	46± 2	98		33± 1	100	
		500	32± 1	100		40± 2	100	

a Sporelings were assayed against phytoalexins by the solution addition method. Germ tube lengths (GTL) were recorded after 18h and a percentage inhibition (I) of germ tube growth calculated. ED_{50}s were determined from plots of phytoalexin concentration (µM) against percentage inhibition of germ tube growth.

b Mean ± SEM.

E. Antifungal activity of flavonoid and isoflavonoid compounds structurally related to the hydroxyflavan phytoalexins

In an attempt to define a structural basis for the antifungal activity of hydroxyflavan phytoalexins, a range of flavans, isoflavans, flavones and flavanones were assayed against B. cinerea and C. herbarum in sporeling assays (solution addition method) and in TLC plate bioassays. Inhibitions of B. cinerea sporeling growth in SPN and in Czapek Dox are given in Tables 3.24 and 3.25 and C. herbarum plate bioassays are illustrated in Plates 3.18 and 3.19. Agreement between the different assays was generally good (Table 3.26).

Flavonoid compounds with antifungal activity were flavone, flavanone, 4-hydroxyflavan and 7-methoxy-4'-hydroxy-8-methylflavan. Of these compounds flavone was strongly antifungal in three sporeling assays, but less so in a TLC plate assay; flavanone was strongly antifungal in a Czapek Dox sporeling assay, and 4-hydroxyflavan and 7-methoxy-4'-hydroxy-8-methylflavan were slightly antifungal in all assays. The flavylium salts of the hydroxyflavan phytoalexins showed strong activity in the Czapek Dox sporeling assay but weak or no activity in the TLC plate assay. Liquiritigenin, only differing from the phytoalexin 7,4'-dihydroxyflavan by a carbonyl group at C-4 in ring C, was inactive in the sporeling bioassays and very weakly active in the TLC plate assay. Flavan compounds with four or five hydroxyl groups (fisetinidol, epicatechin, mollisacacidin and 7,4'-dimethoxyflavan) were all inactive.

The isoflavan analogue of 7-hydroxyflavan (NA37) was antifungal in a TLC plate bioassay with C. herbarum (Plate 3.19).

TABLE 3.24 Comparison of the antifungal activity of a range of flavonoid compounds by bioassay in SPN against sporelings of B.cinerea[a]

Compound[b]	Conc.[c] (µM)	Experiment 1 GTL[d] (µm)	Experiment 1 I (%)	Experiment 2 GTL[d] (µm)	Experiment 2 I (%)
Controls					
SPN		323± 2	-	304± 2	-
SPN + 2% DMSO		310± 2	-	308± 3	-
Flavones					
1. Flavone	50	152± 7	57	144± 8	62
	200	54± 2	92	61± 2	95
2. Chrysin*	50	>300	0	>300	0
(5,7-Dihydroxyflavone)	200	311± 2	0	280± 8	9
Flavanones					
3. Flavanone	50	293±13	6	240± 9	25
	200	207±10	39	199± 7	41
4. Liquiritigenin	50	>300	0	-	-
(4',7-Dihydroxyflavonone)	200	318±14	0	-	-
5. Naringenin	50	>300	0	>300	0
(4',5,7-Trihydroxyflavanone)	200	307±11	1	298± 9	2
6. Poriol*	50	>300	0	>300	0
(6-Methylnaringenin)	200	295±11	5	287± 9	7
Flavans					
7. 4-Hydroxyflavan	50	278±11	15	276± 9	11
	200	248±10	22	237± 8	26
8. 7-Methoxy-4'-hydroxy-8-methyl-flavan	50	309±10	0	273± 8	12
	200	194±10	42	51± 2	98
9. L-Fisetinidol*	50	>300	0	>300	0
(3,7,4',5'-Tetrahydroxyflavan)	200	318±11	0	299± 9	2
10. L-Epicatechin*	50	>300	0	>300	0
(3,5,7,4',5-Pentahydroxyflavan)	200	292±13	6	324± 9	0
11. D-Mollisacacidin*	50	>300	0	>300	0
(3,4,7,4',5'-Pentahydroxyflavan)	200	312±13	0	302± 8	1
Synthetic phytoalexins					
12. (±)-7-Hydroxyflavan	50	137± 6	62	139± 7	63
	100	43± 2	96	52± 2	98
	200	42± 2	96	47± 2	100
13. (±)-7,4'-Dihydroxyflavan	50	293±11	0	309±10	0
	100	264±12	24	242±11	22
	200	218±11	55	60± 2	95

(contd.)

TABLE 3.24 (contd.)

Compound[b]	Conc.[c] (μM)	Experiment 1 GTL[d] (μm)	Experiment 1 I (%)	Experiment 2 GTL[d] (μm)	Experiment 2 I (%)
14. (±)-7,4'-Dihydroxy-8-methyl-flavan	50	227±10	30	239±10	26
	100	161± 7	54	143± 9	62
	200	48± 2	94	50± 2	98

a Compounds were assayed by the solution addition method. Germ tube lengths (GTL) were measured after 18h and a percentage inhibition (I) calculated.

b The source of each compound is given in Section IV.4 of the methods.

c Concentrations were determined by UV absorption at ελmax (Appendix 3) or by weighing known amounts (marked *).

d Mean ± SEM.

TABLE 3.25 Comparison of the antifungal activity of a range of flavonoid compounds by bioassay in Czapek Dox against sporelings of B. cinerea[a]

Compound[b]	Conc.[c] (µM)	Germ tube length (µm)[d]	% inhibition	ED_{50} (µM)
Control (SPN+2% DMSO)		230 ± 11		
1. Flavone	10	156 ± 8	38	
	50	163 ± 6	34	90
	100	125 ± 6	53	
	250	30 ± 2	100	
2. Flavanone	10	201 ± 11	15	
	50	166 ± 7	32	93
	100	121 ± 7	55	
	250	32 ± 2	100	
3. Liquiritigenin	10	>200	0	
	50	>200	0	>250
	100	228 ± 8	1	
	250	231 ± 13	0	
4. Naringenin	10	>200	0	
	50	259 ± 9	0	>250
	100	187 ± 10	22	
	250	185 ± 9	23	
5. 4-Hydroxyflavan	10	203 ± 10	11	
	50	214 ± 10	8	216
	100	178 ± 9	26	
	250	118 ± 6	57	
6. 7-Methoxy-4'-hydroxy-8-methyl-flavan	10	223 ± 10	4	
	50	186 ± 8	22	222
	100	153 ± 9	39	
	250	47 ± 4	53	
7. L-Epicatechin*	10	199 ± 11	16	
	50	195 ± 9	18	>250
	100	153 ± 8	39	
	250	148 ± 9	42	
8. (±)-7-Hydroxyflavylium chloride*	10	79 ± 6	90	
	100	68 ± 5	93	< 10
	500	41 ± 3	100	
9. (±)-7,4'-Dihydroxyflavylium chloride*	10	247 ± 17	34	
	100	217 ± 15	43	126
	500	146 ± 11	67	

(contd.)

TABLE 3.25 (contd.)

Compound[b]	Conc.[c] (μM)	Germ tube length (μm)[d]	% inhibition	ED_{50} (μM)
10. (±)-7,4'-Dihydroxy-8-methyl-flavylium chloride*	10	207 ± 10	46	
	100	197 ± 12	50	100
	500	115 ± 8	77	

Footnotes as in Table 3.24.

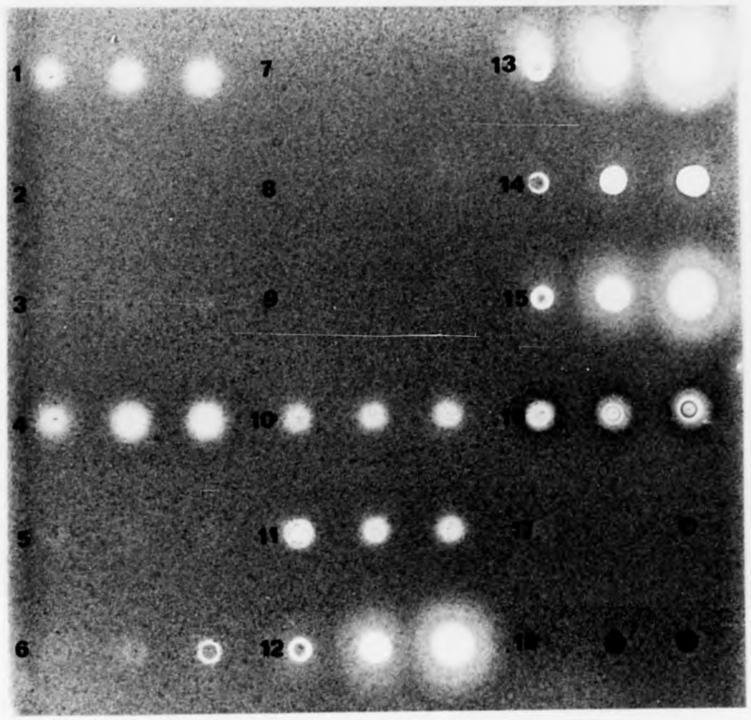

PLATE 3.18 TLC plate spot bioassay of flavonoids. Each compound was assayed at three concentrations (1, 5, 10 mM) in 20µl MeOH.

1. Flavanone; 2. naringenin; 3. poriol; 4. flavone; 5. chrysin; 6. liquiritegenin; 7. epicatechin; 8. fisetinidol; 9. mollisacacidin; 10. 4-hydroxyflavan; 11. 7-methoxy-4'-hydroxy-8-methylflavan; 12. 7,4'-dihydroxy-8-methylflavan; 13. 7-hydroxyflavan (PA9); 14. 7,4'-dihydroxy-flavan (PA6); 15. 7,4'-dihydroxy-8-methylflavan (PA7); 16. 7-hydroxyflavylium chloride; 17. 7,4'-dihydroxyflavylium chloride; 18. 7,4'-dihydroxy-8-methylflavylium chloride.

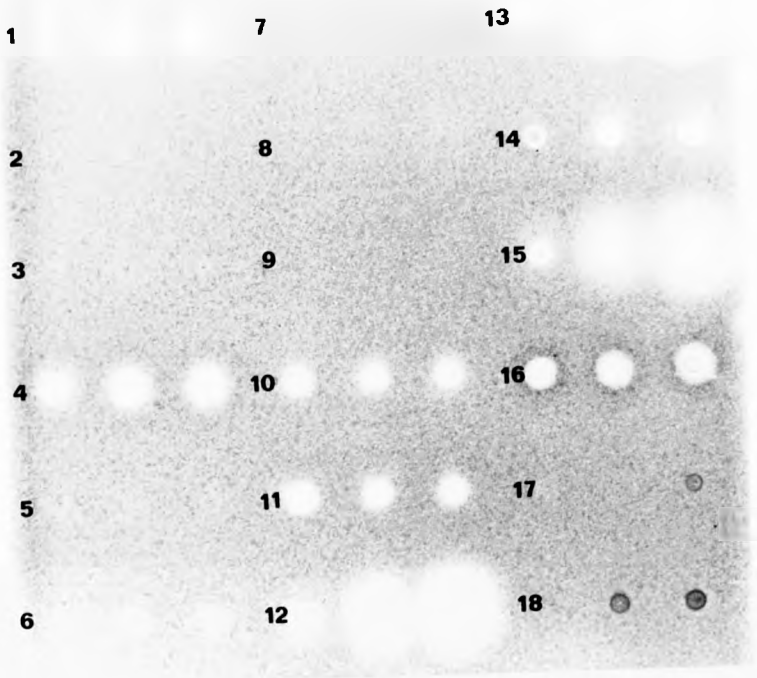
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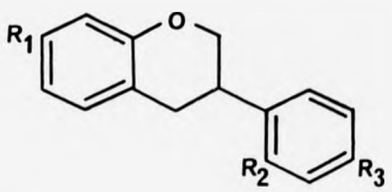
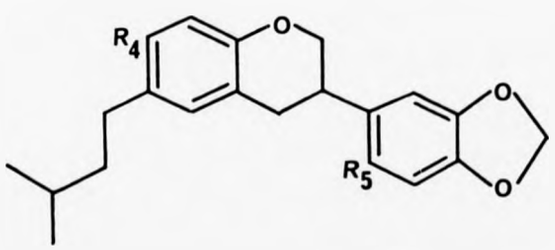
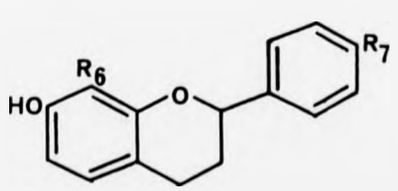
PLATE 3.19 TLC plate spot bioassay of isoflavans. Each compound was tested at three concentrations (1,5,10 mM) in 20µl MeOH or $CHCl_3$. Note that compound 1, the isoflavan analogue of 7-hydroxyflavan, is antifungal. Compound 4 was found to contain major impurities.



1. R_1=OH, R_2=R_3=H (NA37)
2. R_2=OH, R_1=R_3=H (" 38)
3. R_1=R_2=OH, R_3=H (" 39)
4. R_1=OH, R_2=H, R_3=OMe (" 40)
5. R_1=R_3=OMe, R_2=OH (" 41)
6. R_1=R_3=OMe, R_2=OCH_3OCH_3 (" 42)
7. R_1=OMe, R_2=OCH_2OCH_3, R_3=H (" 43)
8. R_1=OH, R_2=OMe, R_3=H (" 44)



9. R_4=R_5=OH (NA45)
10. R_4=OMe, R_5=OH (" 46)
11. R_4=OMe, R_5=OCH_2OCH_3 (" 47)



12. R_6=R_7=H
13. R_6=H, R_7=OH
14. R_6=Me, R_7=OH

narcissus phytoalexins

15. Solvent controls

of isoflavans. Each
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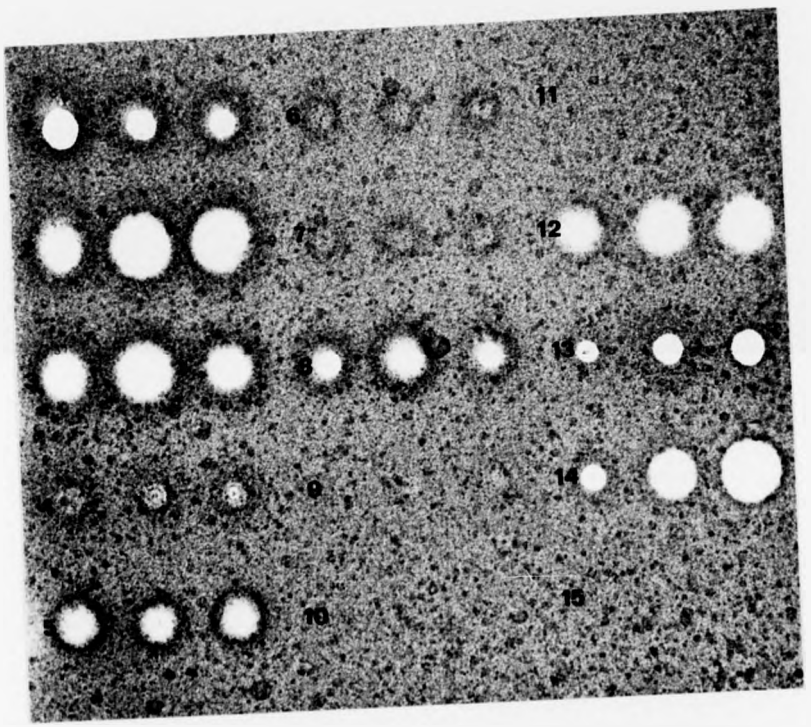

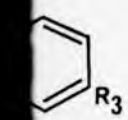

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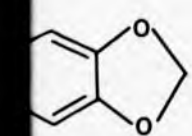

NA45)
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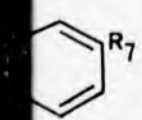

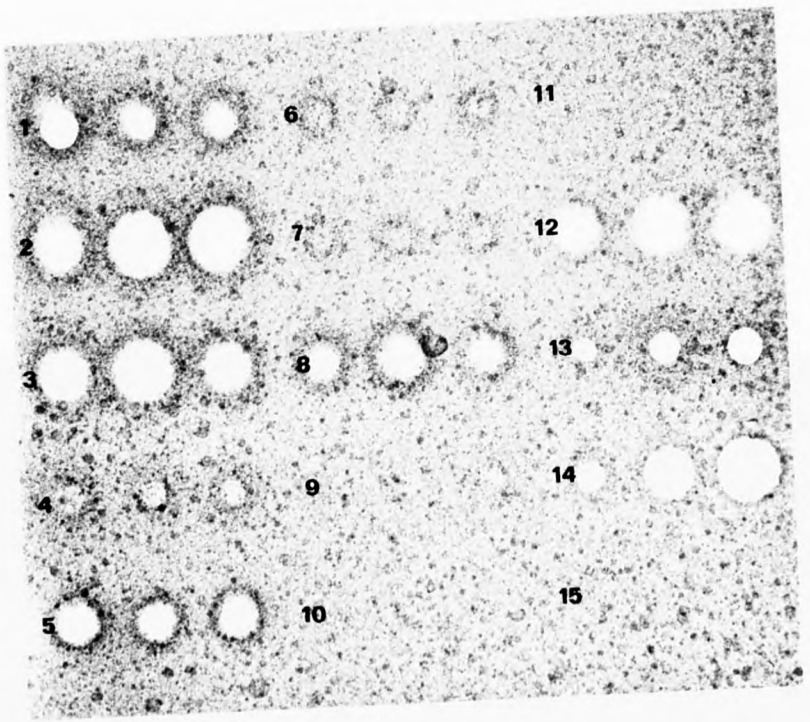
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TABLE 3.26 Summary of the antifungal activities of hydroxyflavan phytoalexins and related flavonoid compounds as revealed in B. cinerea sporeling bioassays and C. herbarum plate bioassays

Compound	Ed_{50} (μM) in B. cinerea sporeling bioassays [a]			Score in C. herbarum plate assays [b]	
	In Czapek Dox	In SPN		20	200 nmoles
		Exp. 1	Exp. 2		
Flavones					
1. Flavone	90	c.50	c. 50	1	1
2. Chrysin	-	>>200	>>200	0	0
Flavanones					
3. Flavanone	93	>200	>200	1	1
4. Liquiritigenin	>>250	>>200	>>200	0	1
5. Naringenin	>250	>>200	>>200	0	0
6. Poriol	-	>>200	>>200	0	0
Flavans					
7. 4-Hydroxyflavan	216	>200	>200	1	1
8. 7-Methoxy-4'-hydroxy-8-methyl-flavan	222	c.200	50-200	1	1
9. 7,4'-Dimethoxyflavan	-	-	-	0	0
10. L-Fisetinidol	-	>>200	>>200	0	0
11. L-Epicatechin	>250	>>200	>>200	0	0
12. D-Mollisacacidin	-	>>200	>>200	0	0
Flavylium salts					
13. (±)-7-Hydroxyflavylium chloride	<100	-	-	1	1
14. (±)-7,4'-Dihydroxy-flavylium chloride	126	-	-	0	0
15. (±)-7,4'-Dihydroxy-8-methyl flavylium chloride	100	-	-	0	0
Synthetic phytoalexins					
16. (±)-7-Hydroxyflavan	-	< 50(98)	< 50	2	4
17. (±)-7,4'-Dihydroxy-flavan	170	c. 200(267)	100-200	2	4
18. (±)-7,4'-Dihydroxy-8-methyl flavan	46	c. 100(124)	c.100	2	4

[a] Where precise ED_{50} values were not obtained the values presented are qualified according to the percentage inhibition of germ tube growth: >>, 0-10% inhibition; >, 10-40% inhibition; c., 40-60% inhibition and <, 60-100% inhibition; -, not determined.

[b] See footnote to Table 3.22.

Several other isoflavans with just one or two hydroxyl groups, or a hydroxyl and a methoxy group, were also active, whereas compounds without a polar group (NA42, NA43) lacked activity (Table 3.28).

The structures of flavonoid and isoflavonoid compounds which I assayed and related compounds reported to possess antifungal activity are described in Tables 3.27 and 3.28. Antifungal activity was not confined to those compounds with a flavan or isoflavan ring structure but was also found in compounds with a C-2 - C-3 double bond (eg. flavone).

Antifungal compounds did not share a common pattern of flavonoid ring substitution; active structures included molecules with various side groups (hydroxyl, methoxy, methyl, carbonyl) substituted at different positions around the ring. The three flavan phytoalexins and many of the other active compounds all possess an hydroxyl group at C-7 in the A ring. However, conversion of C-7 hydroxyl to the less polar methoxy group did not always remove activity (cf. structures 3 and 4 in Table 3.27) and, given the presence of additional polar substituents, the presence of a C-7 hydroxyl did not always confer activity (e.g. chrysin, liquiritigenin, naringenin, epicatechin, mollisacacidin). The only feature common to antifungal flavonoids was the presence of one or two polar groups, but not more, substituted at various positions in the ring.

F. Antibacterial activity of hydroxyflavan phytoalexins, flavone and flavanone

The antibacterial activities of the three hydroxyflavan phytoalexins and two additional flavonoid compounds (flavone

TABLE 3.27 Structure and antifungal activity of flavans, flavones and flavanones

Compound (trivial name)	Position of substitution in the flavonoid ring[a] 3	4	5	6	7	8	2'	3'	4'	Antifungal activity[b]
Flavans										
1. Narcissus PA9					OH					4
2. " PA7					OH				OH	3
3. " PA6					OH	Me			OH	4
4. -					OMe	Me			OH	2
5. -		OH								1
6. Fisetinidol	OH				OH			OH	OH	0
7. Mollisacacidin	OH	OH			OH			OH	OH	0
8. Epicatechin	OH		OH		OH			OH	OH	0
Flavones										
9. Flavone		O								2
10. Chrysin		O	OH		OH					0
Flavanones										
11. Flavanone		O								1
12. Liquiritigenin		O			OH				OH	1
13. Naringenin		O	OH		OH				OH	0
14. Poriol		O	OH	Me	OH				OH	0
15. Betagarin		O	OMe	O-CH_2-O			OMe			*

a The structure and numbering of flavonoid compounds is shown in Fig. 3.16.

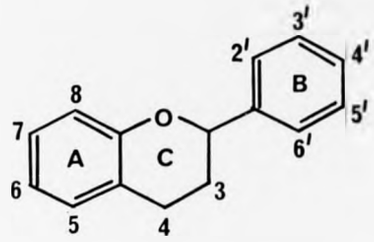
b Antifungal activity of compounds (200 nmoles) was assessed by TLC plate bioassay with C. herbarum (Plate 3.21) on a scale from 0 (inactive) to 4 (highly active); *, reported antifungal in the literature.

TABLE 3.28 Structure and antifungal activity of isoflavans

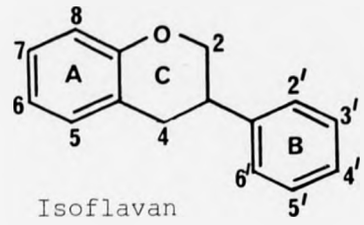
Compound (trivial name)	3	4	5	6	7	8	2'	3'	4'	5'	Antifungal activity
1. NA37					OH						3
2. NA38							OH				4
3. NA39					OH		OH				4
4. NA40					OH				OMe		1
5. NA41					OMe		OH		OMe		2
6. NA42					OMe		OCH_2OCH_3		OMe		0
7. NA43					OMe		OCH_2OCH_3				0
8. NA44					OH		OMe				3
9. Equol					OH				OH		*
10. Demethylvestitol					OH		OH		OH		*
11. Vestitol					OH		OH		OMe		*
12. Isovestitol					OH		OMe		OH		*
13. Sativan					OH		OMe		OMe		*
14. Methoxyvestitol			OMe		OH		OMe		OMe		*
15. Isosativan					OMe		OH		OMe		*
16. Arvensan					OMe		OMe		OH		*
17. Mucronulatol					OH		OMe	OH	OMe		*
18. Isomucronulatol					OH		OH	OMe	OMe		*
19. Laxifloran					OH		OMe	OMe	OH		*
20. Phaseollinisoflavan					OH		OH	5C——O			*
21. 2'-Methoxyphaseollinisoflavan					OH		OMe	5C——O			*
22. 2'-Methoxyphaseollidinisoflavan					OH		OMe	5C	OH		*
23. Astraciceran					OH		OMe	O-CH_2-O			*

a The structure and numbering of isoflavans is shown in Fig. 3.16.

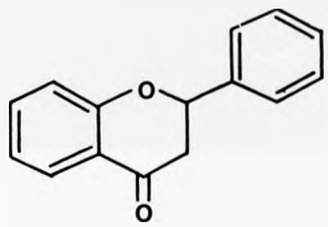
b Antifungal activity of compounds (200 nmoles) was assessed by TLC plate bioassay with C. herbarum (Plate 3.22) on a scale from 0 (inactive) to 4 (highly active). Isoflavans reported antifungal in the literature (*) are also listed; 5C refers to a five-carbon chain or ring.



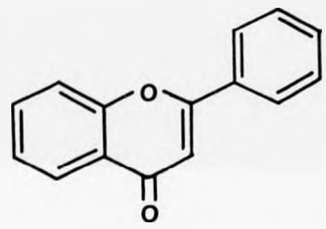
Flavan



Isoflavan



Flavanone



Flavone

FIG. 3.16 Structure and numbering of flavonoid compounds. Note that free rotation abour C-2 - C-1' (C-3 - C-1' for isoflavans) between rings B and C means that positions 2' and 6', and 3' and 5', are identical pairs.

and flavanone) previously shown to possess some antifungal activity, were investigated. The test compounds were assayed against eight Gram-negative and six Gram-positive bacteria by the paper disc method. Streptomycin sulphate was included in the bioassays as a reference antibacterial compound.

Streptomycin was the most active compound, inhibiting all bacteria except Proteus rettgeri (Table 3.29). The hydroxyflavan phytoalexins and flavone were all selectively toxic to Gram-positive bacteria, while flavanone was inactive. The largest inhibition zones were produced by 7-hydroxyflavan (PA9) and 7,4'-dihydroxy-8-methylflavan (PA7) assayed against Corynebacterium fascians and Corynebacterium betae (Table 3.29). The inhibition zone in a plate of C. fascians produced by 50µg of 7-hydroxyflavan was similar to that produced by streptomycin (Plate 3.20).

In general, the realtive activities of the hydroxyflavans against C. herbarum and B. cinerea (7-hydroxyflavan > 7,4'-dihydroxy-8-methylflavan > 7,4'-dihydroxyflavan) was similar against Gram-positive bacteria (Table 3.29). The greater antimicrobial activity of flavone than flavanone previously found in fungal assays was demonstrated even more clearly in assays against bacteria.

TABLE 3.29 Antibacterial activity of 50µg samples of 7-hydroxyflavan (PA9), 7,4'-dihydroxyflavan (PA6), 7,4'-dihydroxy-8-methylflavan (PA7), flavone (F1), flavanone (F2) and streptomycin sulphate (SS)

Bacterium	Source	Gram reaction	Area of Inhibition (mm^2)[a]					
			PA9	PA6	PA7	F1	F2	SS
Erwinia carotovora var. *atroseptica*	G.D. Lyon[b]	-	0	0	0	0	0	212
E. carotovora var. *carotovora*	NCPPB968[c]	-	0	0	0	0	0	302
Proteus rettgeri	SCC[d]	-	0	0	0	0	0	0
Pseudomanas phaseolicola	NCPPB1321	-	0	0	0	0	0	483
P. syringae	NCPPB281	-	0	0	0	0	0	352
Vibrio anguillarum	SCC	-	0	0	0	0	0	214
Xanthomonas phaseoli	NCPPB2064	-	0	0	0	0	0	483
X. phasedi var. *vignicola*	NCPPB2059	-	0	0	0	0	0	302
Bacillus megaterium	SCC	+	29	22	67	42	0	302
Corynebacterium betae	G.D. Lyon	+	270	58	173	46	0	727
C. fascians	NCPPB1675	+	610	50	120	36	0	776
Micrococcus lysodeikticus	SCC	+	22	0	25	0	0	727
Microbacterium phlei	SCC	+	32	10	39	29	0	633
Streptomyces scabies	NCPPB2537	+	35	43	58	43	0	907

a Area of inhibition = area of total inhibition - area of disc; each value is the integer mean of two experiments; no zones of inhibition developed around control discs.

b Dr. G.D. Lyon, SHRI, Invergowrie, Dundee.

c National Collection of Plant Pathogenic Bacteria, Harpenden, Herts.

d Stirling University Culture Collection.

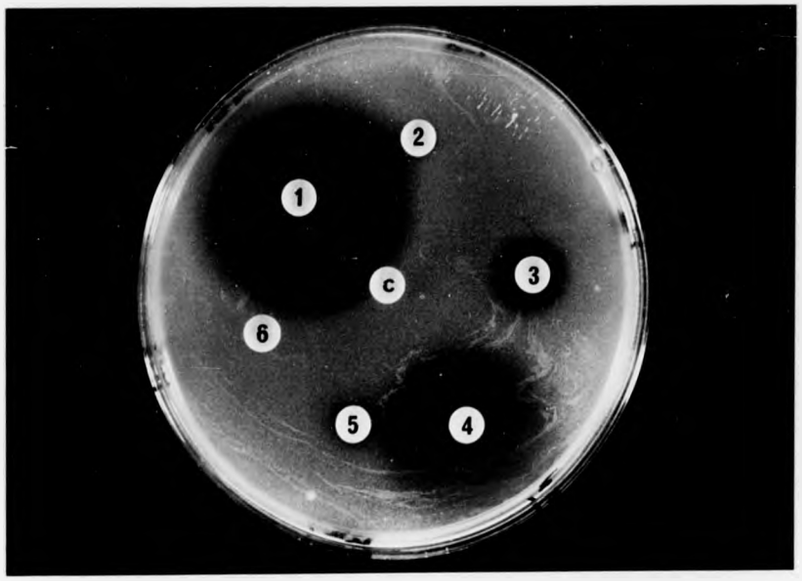
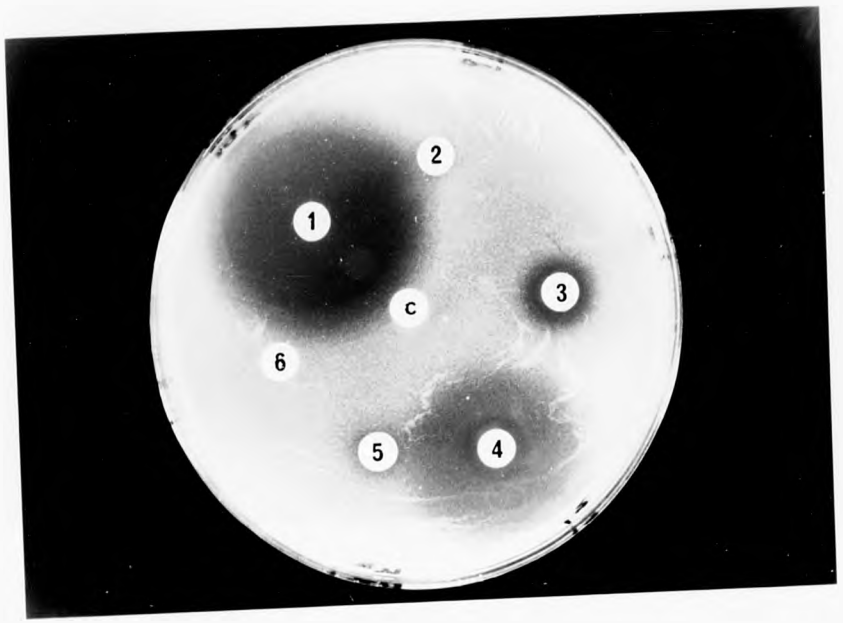


PLATE 3.20 Inhibition of growth of Corynebacterium fascians by 7-hydroxyflavan (1), flavone (3) and streptomycin sulphate (4); 7,4'-dihydroxy-8-methylflavan (5) caused slight inhibition, 7,4'-dihydroxyflavan (6) and flavanone (2) were inactive at the concentration tested (50µg). Solvent alone was applied to the central disc, c.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DISCUSSION

SECTION I

THE CAUSE OF SMOULDER AND THE INFECTION OF NARCISSUS BY SPECIES OF *BOTRYTIS*

1. *Identification of* B. narcissicola *and* B. cinerea

Botrytis narcissicola and *B. cinerea* were not readily identified from the morphological features of sporulating mycelium; small differences in morphology between the two species being confounded by variation between and within isolates, particularly of *B. cinerea*. However, the size and pattern of sclerotia produced by isolates of *Botrytis* grown under identical conditions on PDA were found to be consistent and the types produced by the two species differed sufficiently for sclerotia to be used as a simple means of identification (Plate 1.1). Sclerotia found in infected narcissus tissue in the field were variable, and not indicative of the species.

Isolates of *B. narcissicola*, initially identified on the basis of sclerotial morphology, were clearly distinguished from isolates of *B. cinerea* by their greater pathogenicity towards detached narcissus tissue from mycelial inocula; differentiation was particularly clear in bulb scales.

2. *Association of* B. narcissicola *and* B. cinerea *with smoulder symptoms*

B. narcissicola was the species most commonly found associated with the typical smoulder symptom - the dark brown

leaf lesion. Previous reports on the isolation of *B. cinerea* from narcissus were substantiated but this species was isolated only occasionally from lesions in otherwise healthy shoots, being found mainly in senescent tissue. Evidence was also produced to support the contention of Moore (1979) that some of the sclerotia occurring in outer bulb tissue are *B. cinerea* rather than *B. narcissicola*.

The association of *B. cinerea* with a *Botrytis* species of more restricted host-specificity in one disease syndrome is not unique to narcissus. For example, in chocolate spot of bean (*Vicia faba*) *B. cinerea* and *B. fabae* may cause similar symptoms (Wilson, 1937; Sundheim, 1973) and either *B. cinerea* or *B. squamosa* may be isolated from onion leaf blight symptoms (Hancock and Lorbeer, 1963). The relative roles of *B. cinerea* and the host-specific pathogen in causing disease symptoms appear to vary with the disease. On field bean leaves *B. cinerea* may occasionally cause spreading lesions from conidial inocula (Sundheim, 1973) although lesions spread less rapidly than those caused by *B. fabae* (Purkayastha and Deverall, 1965a; Mansfield and Deverall, 1974). On onion leaves *B. cinerea* conidia cause a superficial fleck whereas lesions formed by *B. squamosa* are rapidly followed by leaf blighting (Hancock and Lorbeer, 1963). From the identification of isolates found associated with narcissus and from the results of pathogenicity tests it would appear that *B. narcissicola* is the major cause of smoulder.

3. Specificity of Botrytis - narcissus interactions

In a comparison of the pathogenicities of six Botrytis spp. towards narcissus it was found that spreading lesions were consistently formed only by mycelial inocula of B. narcissicola isolates. This indicates that narcissus may be nutritionally most suitable for the growth of B. narcissicola. The ability of B. cinerea to cause some spreading lesions in detached narcissus tissue was perhaps predictable from knowledge of its wide host range and occasional association with narcissus. The ability of B. tulipae occasionally to cause spreading lesions in narcissus may reflect some similarity between narcissus and tulip tissues. To test this hypothesis it would be interesting to examine the pathogenicity towards narcissus of B. galanthina, a species usually found on snowdrop (Galanthus spp.), a plant in the same family, Amaryllidaceae, as narcissus. Harrison and Fox (1979) recently isolated B. galanthina from raspberry petals. The host range of B. narcissicola was not studied here but reports by Klebahn (1907) and Dowson (1926) suggest that, although not exclusive, the range is restricted. They found that B. narcissicola attacked snowdrop but not iris, crocus, tulip, hyacinth or scilla plants. Thus, both host and pathogen components of the B. narcissicola - narcissus interaction appear to possess a high level of specialisation; it is interesting to speculate whether or not this is the result of a long period of coevolution.

Isolates of B. cinerea collected from narcissus showed no greater virulence towards narcissus than did isolates collected from a range of plant species. The isolates which

most frequently gave rise to a few spreading lesions from mycelial inocula were obtained from field bean and carrot. There is therefore no evidence to support the erection of a forma specialis of B. cinerea adapted to narcissus. Isolates of B. narcissicola did not appear to differ greatly in their pathogenicities towards narcissus; some differences were noted, particularly with conidial inocula and on leaf tissue, but these were not consistent.

4. Conditions influencing artificial infection of narcissus by Botrytis

A. Nature of the inoculum

Previous workers have noted an apparent failure of B. narcissicola conidia to infect either healthy narcissus plants (Klebahn, 1907; Dowson, 1924; Gray, 1971) or detached narcissus tissue (Gray, 1971). The results obtained here, using defined conidial inocula and incubation conditions, largely support these observations. Even at a concentration of 10^6 spores/ml, conidial inocula, in the abscence of nutrients, failed to infect young, healthy tissue. Failure to infect from conidial inocula is unusual for a specialised Botrytis species with a restricted host-range (another exception is B. galanthina on snowdrop leaves; Massee, 1901). The apparent failure of B. narcissicola to infect narcissus leaves prompts the question as to how the disease is spread. Infection by ascospores is one possibility, but these are rarely found in the field. Other inocula, which may be important in the field, are mycelium in a saprophytic base (e.g. dead flowers, leaf debris) or conidia supplemented with pollen. Infection of damaged tissue may also be

important. The limited lesions, which frequently result when conidial inocula of B. narcissicola fail to invade, may retain the fungus in a quiescent phase. The roles of different types of inoculum in the disease cycle are discussed more fully in Section II.

B. Age of the host tissue

The observation that more spreading lesions develop in detached bulb tissue than in leaves could have several explanations. Unlike bulb tissue, narcissus leaves have a thick, waxy cuticle; the physical and/or chemical barrier offered by this layer may be sufficient for the leaf to resist some attempts at penetration. Another possibility is that lesion frequencies relate to tissue carbohydrate or sugar levels. The storage tissue of fleshy bulb scales and leaf bases are rich in carbohydrate reserves while the leaf has a lower carbohydrate level, considerable fibrous material and large air spaces. Although no sugar levels were measured, Horsfall and Dimond's classification of Botrytis spp. as high sugar pathogens could perhaps be invoked to explain the high susceptibility of the youngest bulb tissue (first generation unit), of young leaves (tested at emergence in February) and the lower (younger) leaf half. Similarly the low susceptibility of the outermost, oldest bulb tissue may result from a low sugar content. The increase in susceptibility of bulb tissue with shoot growth is difficult to explain on Grainger's hypothesis - Cp, the carbohydrate level of the bulb, would be expected to fall. The greatest change in leaf susceptibility was found with senescence. Again this is not expected from Grainger's theory; an increase in leaf sugar levels with senescence appears unlikely.

The more plausible explanation, probably for all the observed changes in susceptibility with tissue senescence, is a decline in the response of the host mechanisms of active resistance (Section III).

C. Wounds

Klebahn (1907), Dowson (1924) and Gray (1971) all noted that tissue wounding was required for colonisation of narcissus by B. narcissicola. Dowson obtained successful infection by inoculating B. narcissicola mycelium or spores into wounded leaves or flower stalks; the nature of wounding was not indicated. Gray observed infection of detached leaves from sclerotia (which germinated to produce conidia) after scraping away the cuticle or making a cut in the leaf. The results presented here show that only slight damage (piercing the cuticle with a needle) is sufficient to allow some infection from B. narcissicola conidia. Insect damage may be the natural parallel of pricking with a needle and in this context it is interesting that Gray and Shiel (1975) have suggested that the bulb scale mite (Steneotarsonemus laticeps) may have a role in the establishment of smoulder infections by damaging bulb tissue during feeding. Severe damage (freezing tissue) prior to inoculation was required before B. cinerea conidial inocula caused any spreading lesions. This suggests that B. cinerea needs to establish itself in a saprophytic base, developing a high inoculum potential (sensuo Garrett, 1956) before invading adjacent healthy tissue. Infection of many host plants by B. cinerea commonly begins with the fungus in a saprophytic phase (Jarvis, 1977). In commercial fields of narcissus,

particularly after flower picking, considerable damage to the foliage is commonly observed. The roles of such wounds in smoulder epidemiology are discussed in Section II.

D. Nutrients

The striking difference in the ability of B. narcissicola to infect detached narcissus tissue from mycelial inocula and from conidial inocula probably represents a difference in inoculum potential, i.e. the well developed mycelium on V8-juice agar has a much greater energy of growth available for infection of the host than a suspension of conidia in SDW. The results presented in Section 1.4C indicate that the addition of pollen, V8 juice, Czapek Dox and to a lesser extent SPN, sufficiently raised the inoculum potential of B. narcissicola conidial suspensions to permit invasion. Corresponding increases in the inoculum potential of B. cinerea conidial suspensions were insufficient to allow infection; even from mycelial inocula on V8 juice agar B. cinerea typically failed to infect.

The nature of the active principle in pollen responsible for increasing infections by Botrytis appears from previous studies to be more than a simple carbohydrate (Chou and Preece, 1968; Warren, 1972; Deramo, 1980). Glucose and fructose were found to be the main sugar components of aqueous broad bean pollen extracts but addition of these sugars, either singly or as a mixture, was not as effective as aqueous pollen extract in stimulating the formation of spreading lesions (Chou and Preece, 1968). On narcissus I found that 50mM glucose increased limited lesion formation but neither B. narcissicola nor B. cinerea initiated spreading lesions.

By contrast, Purkayastha and Deverall (1965) found that 1% glucose (c. 50mM) caused an increase in spreading lesion formation from B. fabae conidial inocula on bean leaves, as well as increasing limited lesion formation from B. cinerea conidial inocula. The failure of glutamine (1mM), mixed with glucose (5mM) or alone, to induce spreading lesions from conidial inocula on narcissus tissue supports the results of Chou and Preece (1968) who found that removal of the α-amino acids from broad bean pollen extracts did not lead to a reduction in their potency. Amino acids would appear to be a minor component, if at all, of the virulence enhancement principle in pollen.

On narcissus I found V8 juice (a mixture of tomato and vegetable juices) to be an effective stimulant of B. narcissicola infections from conidia, a 10% solution (pH6) having an activity similar to that of a pollen suspension at 10^6 grains/ml. Last (1960) and Chou and Preece (1968) found that orange juice increased the percentage of successful infections by B. fabae and B. cinerea on bean leaves. Undiluted orange juice and a suspension of pollen grains (one anther unit, equivalent to c. 0.25×10^5 grains/ml) both resulted in c. 90% of B. cinerea inoculations on bean leaves becoming aggressive. On narcissus bulb tissue Czapek-Dox solution stimulated limited lesion formation by B. cinerea and spreading lesion formation by B. narcissicola. Fokkema (1971) has shown that Czapek-Dox mixed with yeast extract (1% w/v) was as effective as rye pollen in stimulating Drechslera sorokiniana infection of rye.

The nature of the principles in pollen, fruit juices and

other mixtures responsible for virulence enhancement of Botrytis spp. merits further study; examination of how virulence enhancement is achieved might help to elucidate the reasons for differential pathogenicity of two Botrytis spp. on the same host.

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SECTION II

SMOULDER EPIDEMIOLOGY

1. Symptoms

A. Cause

Botrytis narcissicola was the Botrytis species most commonly isolated from dark brown lesions in leaves, the symptom typical of primary infection. Botrytis cinerea was found occasionally, but was more commonly isolated from dying or dead tissue. The failure to isolate B. cinerea frequently from spreading lesions supports the conclusion from pathogenicity tests, that B. narcissicola is the major cause of primary smoulder symptoms.

The frequent isolation of B. narcissicola from rusty-brown flecks, and less commonly from black-brown streaks, strongly suggests that these secondary symptoms are an important part of the smoulder syndrome. The location of brown flecks on leaves adjacent to spreading, sporulating lesions supports the view that they are caused by conidia of B. narcissicola. Although flecking developed only rarely in leaves (of cv. Sempre Avanti) artificially inoculated with conidia, this symptom was observed in preliminary inoculation experiments of several plants, cv. Golden Harvest.

The isolation of B. narcissicola from sickle-shaped leaves suggests this symptom may also be part of the smoulder syndrome. The sickle shape may arise because of asymmetric growth caused by a lesion on one margin at the leaf base (below ground); isolation of the fungus from tissue without an obvious lesion indicates a symptomless phase of growth.

Sickle-shaped leaves can also result from attack by the bulb scale mite (Steneotarsonemus laticeps) but infection rarely occurs on narcissus grown in the field and is usually accompanied by a characteristic saw-edged flower stalk (Winfield, 1970).

Leaf distortion was frequently associated with dark brown lesions, usually near the leaf tip, and so appeared to result from infection by B. narcissicola. However, distortion is also a common symptom of plants infected by the stem eelworm (Ditylenchus dipsaci). The distorted leaves that I observed, however, lacked the swellings ('spickels') characteristic of eelworm infection (Morgan, 1970).

The increase in grassiness observed in the plot of Verger paralleled increases in characteristic smoulder symptoms. It is therefore possible that grassiness may be caused by B. narcissicola rotting the main shoot and thereby stimulating lateral bulbs to produce numerous thin, small leaves.

An interesting symptom observed in the experimental planting at SHRI, in commerical plantings at Cushnie Farm, and also following inoculation of field-grown plants, was that of premature leaf death without the prior development of obvious lesions on affected leaves. Narcissus white streak virus can result in an early decline (McWhorter, 1939), but before senescence the virus causes purple longitudinal streaks which later turn white and coalesce. It therefore seems that premature leaf death may indeed result from infection by B. narcissicola. Perhaps optimal

conditions for fungal growth result in rapid development of a smoulder lesion, leaf withering and death. Alternatively, it may be that with the onset of senescence latent infections spread, causing an accelerated necrosis.

The isolation of B. narcissicola from apparently healthy shoot tissue (Table 2.2) was surprising, suggesting a symptomless or latent phase of infection. Symptomless infection has been recorded for species of Botrytis in other host plants, a good example being that of B. allii in onion leaves (Maude and Presley, 1977). However, after clearing with MeOH and chloral hydrate and staining with trypan blue, I failed to observe mycelium in portions of apparently healthy narcissus leaves, even when the fungus was isolated from adjacent pieces of leaf tissue. Assuming that no contamination occurred while plating out, it would appear that B. narcissicola was present in discrete infections which lacked macroscopic symptoms, i.e. latent infections. Jarvis (1977) suggested that latent infections may be common in diseases caused by species of Botrytis. He listed infections where there was some evidence for latency and included infection of narcissus bulbs and flower petals by B. narcissicola. Botrytis narcissicola latent in narcissus leaves would have to progress and infect bulb tissue to be of importance in the seasonal carryover of smoulder. Premature leaf senescence observed following inoculation of healthy leaves with conidia of B. narcissicola supports the hypothesis that latent infections do indeed eventually spread. The fungus was also isolated frequently from dead leaves and flower stalks in commercial plantings, again possibly resulting from latent infections

turning aggressive although the possibility of infection occurring during senescence cannot be excluded. Latent infections in narcissus leaves may thus be the initial stage of a hidden route leading to bulb infection, additional to the more obvious spreading of lesions down stalks and leaves.

Planting site appeared to influence the frequency with which B. cinerea was associated with narcissus. Plantings at Cushnie Farm, from which samples were collected in 1979, were severely infected with smoulder and inoculum of B. narcissicola was abundant. In contrast, at SHRI in 1978 the plot of cv. Verger had a relatively low frequency of smoulder and adjacent plots of field bean and strawberry probably supplied a large inoculum of B. cinerea. These differences in inoculum might have accounted for (1), the isolation of B. cinerea from leaf tip lesions only at SHRI (2), the isolation from dead flower heads of B. cinerea at SHRI and of B. narcissicola at Cushnie Farm and (3) the failure to isolate B. cinerea from rotting flower stalks at Cushnie Farm when artificial inoculation experiments indicate that B. cinerea may cause such a symptom. Similarly, although only B. narcissicola was isolated from flower spots, it would be surprising if B. cinerea could not cause a similar symptom.

The presence of B. cinerea sclerotia in the outer scales of some bulbs is difficult to explain considering the inability of B. cinerea to infect healthy bulb tissue from mycelial inocula. Presumably B. cinerea in the base of dying leaves is able to colonise fleshy scales as they die and turn papery. In brown stain diseas of onion, B. cinerea infection is confined to the outer, papery bulb scales (Clark and Lorbeer,

1973). The lack of frequent lesions or B. narcissicola infection in the outer, fleshy scales of narcissus bulbs is in marked contrast to B. tulipae infection of tulip bulbs in the fire disease (Price, 1970a; Doornik and Bergman, 1971). The grey patches found in fleshy narcissus bulb scales probably result from bruising.

B. Incidence

The incidence of primary smoulder symptoms was low in first year plantings, both experimental and commercial, and considerably higher in bulbs grown for two years or more. This feature of narcissus smoulder has been noted previously (McWhorter and Weiss, 1932; Gray and Shiel, 1975) and strongly indicates secondary spread of the disease by conidia. A gradual increase in the number of clusters with a primary symptom, from March to May, probably results from delayed emergence of some infected shoots.

The incidence of secondary-infection symptoms rose sharply late in the growing season, after flowering and as leaves were approaching senescence. Infection of only dying leaves by conidia is in agreement with the results of pathogenicity tests on tissues of different age, described in the first chapter.

2. The origin and development of primary symptoms in shoots

A. Infected bulbs

The presence or absence of botrytis sclerotia in the outer bulb scales was found to be of little value in predicting which plants would show smoulder symptoms at emergence. A similar conclusion was reached by Beaumont et al. (1936),

concerning sclerotia on tulip bulbs and the appearance of infected shoots. Although the presence of sclerotia on narcissus undoubtedly indicates fungal infection, the species involved may be B. cinerea rather than B. narcissicola, and sclerotia in the outer scales are no indication that other bulb tissue, at the neck in particular, is infected. Conversely, bulbs without sclerotia may nevertheless be infected with B. narcissicola.

The failure of VT bulbs to emerge after inoculation at the neck with B. narcissicola indicates that an actively growing mycelium either within bulb tissue or on the bulb surface, can readily infect and rot the emerging shoot. If larger bulbs had been used the damage caused to emerging leaves may have been less and some typical primary symptoms produced. Larger bulbs (12-14cm) which were wound-inoculated did produce shoots with typical primary symptoms. The relatively low success of B. narcissicola in causing shoot infection after wound-inoculation, compared with the prevention of all shoot emergence after surface-inoculation, may also reflect a difference in the infection route taken to reach the shoot. In wound-inoculated bulbs B. narcissicola might grow towards the shoot slowly, invading inwards through scale tissue, whereas on unwounded bulbs the fungus might grow quickly towards the bulb neck over the surface scales. The greater frequency of shoots with primary symptoms from bulbs wound-inoculated at the neck than at the base indicates that position of infection in a bulb could be an important factor in determining whether or not a plant develops shoot infection.

As shoots emerge through an infected bulb neck, that

is, between the leaf sheath and flower stalk bases of previous seasons, first the sheath and then the enclosed leaves or flower bud are infected. Infection failing to transfer, either from bulb to sheath or from sheath to shoot, would explain why a plant, apparently healthy one year, may produce shoots with primary symptoms the next (and vice-versa). Simultaneous infection of leaves within a neck would also explain the occurrence of fused leaves and of lesions at a similar height on several leaves in the same shoot.

The results I obtained when investigating factors influencing the production of primaries from infected bulbs were largely inconclusive. A microclimate in the bulb neck which favours development of an active mycelium but slow shoot emergence should enhance the chance of shoot infection and the subsequent appearance of primary symptoms. One might therefore expect that infection would be most probable in the shoots arising from deeply planted bulbs. However, I found that sheath lesions and primary symptoms were most frequent in the shoots which emerged quickly from shallowly planted bulbs.

Again, the growing medium would be expected to have some influence on the frequency of primaries arising from infected bulbs. Although smoulder was noted in VT bulbs grown in a moist compost (at ESCA) and not in the same clones grown in a drier compost (at NOSCA), I failed to find significant differences in the incidences of primaries arising from infected bulbs grown in peat, sand and loam.

Shiel (pers. comm.) has recently shown that within a given planting primary symptoms are often found at the same relative height on shoots of different plants. From the foregoing, his observations could be explained by postulating that all bulb necks within the planting are similarly predisposed to allow infection of emerging shoots at the same stage of development.

It would be interesting to investigate more closely the effect of temperature, relative humidity and other factors that vary with depth of planting and type of growing medium on the transfer of infection from bulb to shoot. My results suggest that infection often transfers from bulb to sheath but no further.

B. Infested soil

A small but significant increase in the number of primaries was found when shoots grew through soil infested with bulb scale debris bearing botrytis sclerotia. In a subsequent experiment, with bulbs grown in pots, the failure of isolated B. narcissicola sclerotia to cause typical primary symptoms was therefore puzzling. Where no foliage occurred, B. narcissicola may have rotted shoots as they arose; the finding of sclerotia in bulb necks at lifting supports this view. It would have been interesting to leave these bulbs to grow for another year and to examine the shoots for primary symptoms in a second season. It may be that B. narcissicola usually spreads in the bulb the first season, after infection from sclerotia, and only causes typical primary symptoms the following season. Where healthy shoots were produced, despite the presence of B. narcissicola sclerotia or debris

in the soil above bulbs, it is possible that (1), growing shoots failed to contact the inoculum (2), attempted infection was unsuccessful or (3) sclerotia germinated and died before shoot development.

Botrytis narcissicola sclerotia buried in soil produce conidiophores, the most common mode of germination for sclerotia of Botrytis spp. (Jarvis, 1977). It is probably germ tubes from conidia which infect emerging narcissus shoots or the bulb. The thin sheath tissue surrounding the shoot would seem to be the most suitable site for infection by conidia. If B. narcissicola becomes established within the sheath it may then cause primary symptoms or grow downwards into the bulb neck. Thus, sclerotia of B. narcissicola or debris buried in the soil may cause primary symptoms, probably with infection of the sheath as an important stage again. However, the overall evidence is more in favour of infected bulbs being the major source of smoulder outbreaks.

3. Secondary infection

A. Wounded tissue

Rotting flower stalks, spreading lesions in broken leaves, and lesions in unwounded leaves adjacent to rotting stalks, were all observed frequently in commercial fields of narcissus shortly after flower picking. In artificial inoculation experiments, B. narcissicola readily invaded wounded leaves and flower stalks. The ability of the fungus to invade wounded leaves was similar irrespective of whether they were attached to or detached from the parent plant. In attached leaves, the unequal lesion spread from wound

sites, greater towards the leaf tip than the leaf base, may have resulted from production of a toxin by the fungus.

Rotting of the ends of cut flower stalks by B. cinerea was unexpected. However, the faster rate of rotting and the more abundant sporulation of B. narcissicola showed it to be the better pathogen of narcissus. The eventual development of rots in water-inoculated stalks probably resulted from conidia released from adjacent stalks inoculated with B. narcissicola. When examined the following spring, water and B. cinerea inoculated plants gave rise to some shoots with primary smoulder symptoms, but in both cases the number was significantly less than from plants inoculated with B. narcissicola (Table 2.16). Spreading lesions in leaf bases were probably initiated as rotting stalks and adjacent leaves came into contact near the soil surface.

B. Unwounded tissue

Healthy leaves of field-grown plants were not readily infected when inoculated by spraying with B. narcissicola conidia in SDW. Although more leaf tip lesions developed in plants inoculated with B. narcissicola conidia than in controls, the relatively large number of lesions in the latter indicate that some may have resulted from damage, possibly caused by the polythene bag incubation treatment. The development of fleck lesions on some leaves inoculated with water or V8 juice alone was also unexpected; spray drift between treatments may be the explanation.

Inoculating healthy leaves with B. narcissicola conidia caused few obvious symptoms. However, it may have caused

symptomless infections which later progressed to cause the observed accelerated leaf death.

In contrast to the laboratory experiments on detached leaves, inoculating field-grown plants with B. narcissicola conidia in V8 juice did not cause spreading lesions. A similar conflicting result was the failure of B. narcissicola to invade leaves of field-grown plants from mycelium on agar. Possible explanations for these differences are (1), active mechanisms of host resistance were more effective in attached leaves of growing plants or (2), conditions in the field were not favourable to fungal growth.

Spread of smoulder during the growing season involves conidia, produced abundantly on plants with primary symptoms when conditions are suitable. The obvious symptom of secondary infection is a spreading lesion, developing in flower stalks after picking or in wounded leaves. Spreading lesions may also develop in unwounded tissue if nutrients (e.g. pollen) are present in inoculum droplets, or when leaves become senescent. Secondary infection of unwounded tissue by conidia would appear to result usually in a limited, fleck lesion. Mycelial inocula may be responsible for some disease spread, as for example, when rotting stalks contact adjacent leaves. Although rotting of leaves and flower stalks down to the soil surface may take several weeks, it seems probable that B. narcissicola spreads within lesions to infect leaf and flower stalk bases within the bulb neck.

4. Infection of bulbs

A. Growth of B. narcissicola down infected shoots

Isolating B. narcissicola from the bulbs of plants with secondary symptoms is good evidence for growth of the fungus through shoot tissue into the bulb neck. Stalks in particular afford an easy route into the bulb after flower picking. Botrytis narcissicola typically caused a stalk rot, rather than a symptomless infection as suggested by Jarvis (1977).

The fact that B. narcissicola was rarely isolated from fleshy tissue c. 5mm below leaf, sheath and flower stalk abscission zones indicates that bulb tissue is more resistant than shoot tissue to invasion. Botrytis narcissicola readily invades the dying outer scales, and such infections would explain the presence of sclerotia within them. More extensive bulb infection (rots) may develop when the environment favours the fungus and/or predisposes the bulb to invasion.

The isolation of B. narcissicola from the bulbs of plants with apparently healthy shoots could have several explanations. For example, bulbs could have been infected at planting but the emerging shoots escaped infection, or, bulb infection may have occurred by a means other than the growth of B. narcissicola down through the shoot from a secondary infection (see below). Again, a spreading lesion in the shoot could progress rapidly down to the bulb and the affected tissue subsequently be lost. Moreover, there may also be symptomless growth through leaves or flower stalks into the bulb.

B. Other sources

Botrytis narcissicola conidia may be washed down leaves to infect tissue at the bulb neck. Sclerotia, mycelium or conidia in the soil may infect the bulb directly. Cross-infection may occur in bulb clusters, particularly where there is a neck rot of the mother bulb. Although none of these routes were investigated, similar pathways leading to below ground infection have been recorded in comparable diseases of other bulbous plants (Moore, 1979).

5. Seasonal carryover

A. In the bulb

Plants infected by B. narcissicola in one season, either natural primaries or following artificial inoculation, frequently emerged with smoulder symptoms the following season. In the bulb, B. narcissicola probably survived as sclerotia in the outer scales or as mycelium in the neck.

The survival of mycelium in bulbs may be influenced by post-harvest practices. Humphrey-Jones (1975) reported that when bulbs of infected plants were lifted and re-planted they emerged the following season without smoulder symptoms. In bulbs stored moist or dry for 6 wks I successfully re-isolated B. narcissicola from restricted lesions in most bulbs following both treatments. It would appear that lifting and dry storage does not eliminate B. narcissicola mycelium from bulbs although it may affect the level of inoculum and hence the ability of the fungus to infect emerging shoots. Botrytis narcissicola may have been re-isolated less frequently from restricted lesions in bulbs if the initial inoculum had been smaller or the storage

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period extended. For seasonal carryover, B. narcissicola probably has to survive in bulb tissue for at least 12-16 wks (July-October) before new leaf tissue appears within the bulb nose.

B. In the soil

In the soil, B. narcissicola may be carried over from one season to the next in the form of conidia, mycelium or sclerotia. The structure most suited to withstanding periods unfavourable to growth is the sclerotium. According to Garrett (1956) the survival of sclerotia depends on their failing to germinate while retaining the capacity to germinate. It seems unlikely that the decrease in survival of B. narcissicola sclerotia I observed can be attributed solely to germination, because the proportion of sclerotia recovered in a germinating state was low, most sclerotia having died before germinating. In contrast, Coley-Smith and Javed (1972) found that the majority of sclerotia of B. tulipae died immediately after conidiophore production in late Winter early Spring, usually within one year of burial. Factors affecting the survival and germination of sclerotia buried in soil have been discussed in a review by Coley-Smith and Cooke (1971).

Although Gregory (1941) induced carpogenic germination of B. narcissicola sclerotia on narcissus debris in south-west England, Gray and Shiel (1975) failed to induce apothecia in Scotland and I also observed only sporogenic germination.

No experiments were performed on the longevity of B. narcissicola conidia buried in soil or the saprophytic

survival of mycelium in debris. However, a limited amount of work has been carried out on the survival in the field of other Botrytis spp., particularly B. cinerea. Park (1955) and Lockwood (1960) noted that conidia of B. cinerea soon disappeared when added to soil and they failed to colonise pieces of plant material below the soil surface. On previously colonised tissue the fungus survived burial for c. 4 weeks (Park, 1955). In dry storage, conidia of B. cinerea, B. tulipae and B. convoluta survived for several months (Bagga, 1967; Beaumont et al., 1936; Mass, 1969). Under field conditions however, with varying temperature and relative humidity, it seems unlikely that Botrytis conidia would remain viable for long periods.

It would appear that B. narcissicola can survive in bulbs between lifting and re-planting as mycelium, although the inoculum may fall to a level insufficient to infect more than a few shoots at emergence. Sclerotia in the soil, and probably on bulbs too, can survive burial for over a year and so carry the fungus over from one season to the next.

6. The disease cycle and control measures

The experimental results discussed in Sections 1-5 suggest a possible disease cycle. This is illustrated in Fig. B which distinguishes stages of the cycle for which I have obtained experimental evidence from other more speculative routes. Suggestions for controlling smoulder are discussed below with reference to the postulated disease cycle.

FIG. B Narcissus smoulder disease cycle

Stages:

1. Infected bulb produces a shoot with a lesion in the sheath.
2. Severely infected bulb rots in the ground.
3. Infected bulb produces a healthy shoot.(NB The bulb may remain infected and produce a shoot with primary infection symptoms the following season.)
4. Healthy bulb produces a healthy shoot.
5. Shoot from a healthy bulb becomes infected from sclerotia or debris in the soil.
6. Infection of leaves and/or flower bud within the sheath.
7. Leaves within the sheath escape infection from the sheath lesion.
8. Conidia produced on infected shoot (primary) infect the shoots of other (healthy) plants (secondary infection).
9. Conidia washed down the shoot into the bulb neck; healthy bulb becomes infected.
10. Further cycles of secondary infection from conidia.
11. Mycelium grows down the shoot into leaf and flower stalk bases in the neck; healthy bulb becomes infected.
12. Deposition of sclerotia or infected debris in or on the soil.
13. Carpogenic germination of sclerotia; ascospores infect healthy shoots.
14. Sclerotia, mycelium or conidia in the soil infect a healthy bulb.
15. Infection spreads during bulb lifting and/or storage.
16. Infected bulbs rot in store (especially neck rot).
17. Infected bulbs, often apparently healthy, become the new planting stock.
18. Cross-infection between bulbs within a cluster.
19. Infection in bulbs is eliminated by a fungicide dip, HWT or storage.

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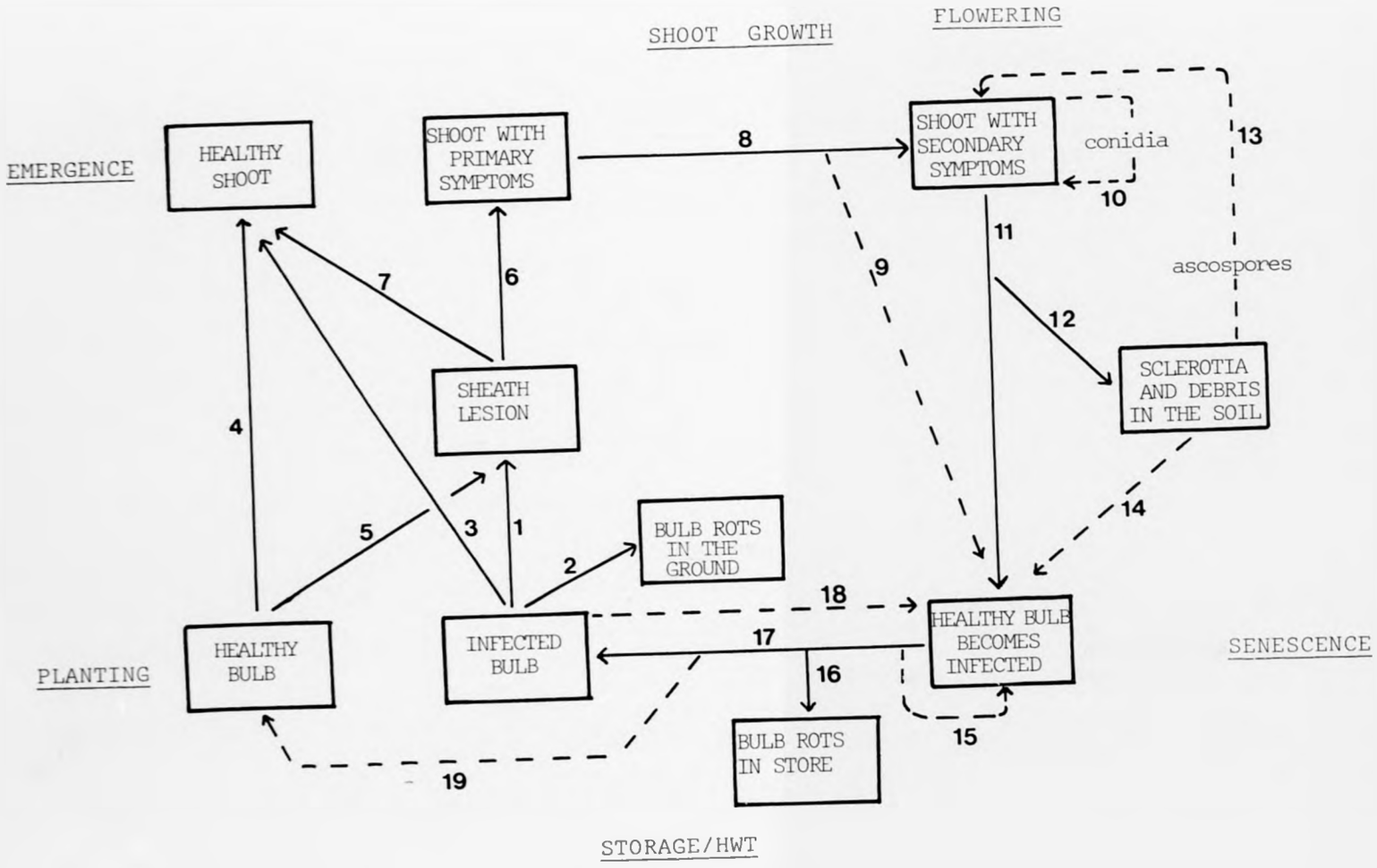

FIG. B.

A. Pre-planting bulb treatments

It is not possible from a dry-bulb inspection to identify all bulbs infected with B. narcissicola. Apparently healthy bulbs, without sclerotia, may carry B. narcissicola mycelium in the neck and apparently diseased bulbs, bearing sclerotia, may simply have a limited infection of the outer scales by B. cinerea. It would therefore be advisable, when purchasing new stocks, for growers to have knowledge of the stocks' health in previous seasons; certified bulbs, particularly VT stocks, are to be preferred.

The incidence of plants with primary symptoms in first year plantings is never high and annual lifting, if economical, would lessen accumulation of infected bulbs. If bulbs were dipped in a systemic fungicide (e.g. benomyl) prior to planting this might reduce the initial incidence and level of bulb infections, so preventing the development of many plants with primary symptoms from bulbs grown for two or more seasons. A reduction in the incidence of tulip fire, also perpetuated mainly in bulbs, has been achieved by dipping bulbs in benomyl after lifting (Price and Briggs, 1974).

B. Cultivation techniques

A rotation of two or three clear years between planting narcissus bulbs in the same land should be sufficient to avoid shoot infections arising from B. narcissicola sclerotia or debris left in the soil. Roguing of plants with primary symptoms will assist in delaying the build up of infection both within a planting and in the bulb stock. However, because not all infected bulbs give rise to shoots

with primary symptoms at the same time, and perhaps not at all in the first year, a crop needs to be rogued several times each year. Where bulbs are grown for more than one season, re-ridging may reduce the typical second year increase in smoulder (Hardwick et al., 1978), possibly by creating an environment unfavourable to B. narcissicola development. The introduction of debris to the soil during cultivation should be avoided.

C. Prevention of spread

As most smoulder results from planting infected bulbs, the most effective means of control may be to aim at preventing any increase in their number. To this end, control of secondary infection, particularly at wound sites after flower picking, would seem to be important. Recent trials (Hardwick, pers. comm.) indicate that stalk infection can be controlled by spraying crops with fungicides after flower picking. For valuable VT stocks a series of fungicide sprays, from leaf maturity to senescence, may be prudent. The ink and leaf spot diseases of iris, caused by Drechslera iridis and Mycosphaerella macrospora respectively, symptoms of which are usually much more frequent in the second season, were controlled by regular spraying with dithiocarbamates after flowering in the first season (Moore, 1979).

The effectiveness of dipping bulbs in a fungicide prior to planting, and spraying with fungicides after flower picking, awaits confirmation from their application in commercial plantings.

SECTION III

MECHANISMS OF RESISTANCE TO *BOTRYTIS* IN NARCISSUS BULBS

1. Fungal growth on bulb and leaf surfaces

The failure of conidia of *B. narcissicola* and *B. cinerea* to cause spreading lesions does not result from poor germination or germ tube growth; fungal development from conidia on narcissus tissue was frequently faster than on glass slides. However, the faster germination and growth on bulb scales than on leaves may explain the more rapid development of symptoms (limited lesions) in the former. Rapid fungal development would also appear to be a feature of successful colonisation, as, for example, when pollen grains were added to suspensions of *B. narcissicola* conidia in SDW.

Because of the numerous factors which affect spore germination and growth on host surfaces, it is of little value to make developmental comparisons with other *Botrytis*/host interactions.

2. Structural resistance

A. Pre-infectional

Evidence for pre-formed structural resistance is usually obtained by correlating the magnitude of a structural feature in different cultivars with the level of infection. The present study of resistance mechanisms in narcissus was confined to bulb tissue of one cultivar - Golden Harvest. However, the fact that bulb scales were largely resistant to infection from *B. narcissicola* conidia after removal of the

epidermis would seem to preclude the cuticle acting as a structural barrier.

In any future search for pre-formed structural resistance in narcissus it may be promising to investigate infection of leaves in relation to their waxiness. Gray (pers. comm.) has suggested that removal of wax by mites increases the level of infection. In addition to acting as a physical feature, perhaps for example by reducing spore deposition, leaf wax may provide pre-infectional chemical resistance (Blakeman and Sztejnberg, 1976; Rossall and Mansfield, 1980).

B. Post-infectional

(i) Nature of the response. Deposits of reaction material and alteration to cell walls, apparently in response to attempted infection, were found in bulb scale epidermal strips following inoculation. A similar response has recently been noted in other plants attacked by species of Botrytis; for example, leaves of Vicia faba at sites of attempted infection by B. cinerea (Mansfield and Hutson, 1980) and in bulbs of tulip and onion following inoculation with non-pathogenic species of Botrytis (Mansfield, pers. comm.). In plants undergoing a resistant response, alteration to the cell wall and deposition of reaction material may be more common than is currently appreciated.

Histochemical tests showed that a polyphenolic polymer, probably lignin, was the major component of new material deposited in narcissus bulbs, particularly in cell walls. 'Lignin' is the term for a complex polymer synthesised from three phenolic alcohols (Gross, 1977) and individual lignins may differ widely in structure and composition. Ride (1975)

for example, found that the composition of lignin newly synthesised following infection differed from that found in healthy wheat leaves. Differences in structure are probably responsible, at least in part, for the failure to find a single, definitive histochemical test for lignin. The chemical specificities of the traditional lignin stains are not identical. The chlorine-sulphite stain is reported to be specific for syringyl units while the Wiesner test (phloroglucinol-HCl) locates cinnamaldehyde end groups (Wardrop, 1971). UV autofluorescence detects wall bound phenolic acid in addition to lignin (Harris and Hartley, 1976). The inconsistencies between tests found when staining narcissus epidermal tissue are therefore not unexpected.

Resistance to cell wall degrading enzymes, organic solvents and conc. H_2SO_4 would support the histochemical evidence for lignin in narcissus. The material(s) could be further characterised by UV spectrophotometry or alkaline nitrobenzene oxidation.

The evidence for callose deposition in narcissus is less substantial. Alcoholic aniline blue, a traditional test for callose (Currier and Strugger, 1956), stained some cell walls at sites of attempted infection while lacmoid, specific for callose or a callose glycoprotein (Reynold and Dashek, 1976) was not taken up.

(ii) Role in resistance. The detection of cell wall alterations in bulb tissue beneath inocula which typically failed to cause spreading lesions indicates that these alterations may be the cause of restricted fungal growth. A more quantitative study of incompatible interactions,

with reference to the timing of wall alteration and limitation of infection, would help to support the role of wall modification in resistance.

A role for cell wall alteration in disease resistance has been partly demonstrated for several other host-parasite interactions. Ride (1980) showed that lignification induced by the non-pathogen B. cinerea increased the resistance of wheat leaf cell walls to fungal degradation. Vance and Sherwood (1976) used cycloheximide to inhibit papillae formation in reed canarygrass, and other species of the Gramineae (Sherwood and Vance, 1980), and found large increases in the level of successful penetration from appressoria of normally incompatible leaf-infecting fungi. Cycloheximide treatment, however, would be expected to inhibit any active host resistance mechanism and not just papillae formation. Aist, Kunoh and Israel (1979) have shown that pre-formed papillae induced by non-pathogens in barley leaves were not breached by haustoria in subsequent inoculations with compatible Erysiphe graminis f.sp. hordei. Henderson and Friend (1979) reported a race-specific increase in phenylalanine ammonia lyase (PAL) activity and deposition of a lignin-like material in potato tubers inoculated with Phytophthora infestans.

(iii) Mode of action. The most obvious means by which cell wall alterations such as lignification might inhibit the progress of fungi is by providing a physical barrier resistant to cell wall degrading enzymes. Ride (1980) showed that in wheat even small amounts of lignin deposited in papillae or altered lateral walls, which may be undetectable by the traditional histochemical tests, can

provide considerable resistance to fungal degradation. He also noted that six fungi pathogenic to wheat had no greater capacity to degrade the lignin deposited on infection than seven non-pathogens. Only Fusarium graminearum was capable of degrading lignin more readily than the non-pathogens, but this attribute seems irrelevant to pathogenicity as in the natural situation F. gramineanum spreads through wheat leaves without inducing large quantities of lignin.

Four additional mechanisms by which lignified walls might inhibit fungal progress were noted by Ride (1978) - (1), providing a barrier to mechanical pressure; (2), restricting diffusion of nutrients and water from the plant to the fungus or enzymes and toxins from the fungus to the plant; (3), the accumulation of phenolic precursors and free radicals formed during polymerisation might affect fungal growth; or (4), hyphae in close proximity to lignifying cells might themselves become lignified. There is some support for the last two mechanisms. Keen and Littlefield (1979) provided evidence that production of coniferyl alcohol and coniferyl aldehyde was responsible for restriction of fungal growth in incompatible flax - Melampsora lini interactions. These compounds could be regarded either as phytoalexins or as lignin precursors. In wheat and rice leaves hyphae in lignifying cells have been observed to take up stains for lignin (Ride, 1978).

3. Chemical Resistance

A. Pre-formed inhibitors (prohibitins, inhibitins and post-inhibitins)

Bulbs appear to be a rich source of secondary plant products, including compounds with antimicrobial activity (Cavallito and Haskell, 1946; Walker and Stahmann, 1955; Hardeggar et al., 1963; Gaumann 1964; Bergman, 1966).

From narcissus bulbs, numerous alkaloids have been isolated (Wildman, 1960; 1968), and some have antimitotic and antiviral activity. However, antifungal activity per se has not been reported. In the present investigation bulbs were initially examined for antifungal compounds present in healthy tissue, released in dying tissue or released from precursors by hydrolysis.

Bioassay of extracts of healthy bulb tissue revealed no prohibitins. The ability of B. narcissicola and B. cinerea to grow on frozen-thawed tissue suggests that neither inhibitins nor post-inhibitins are released as a general wound response. It therefore seems unlikely that any chemical inhibitors other than phytoalexins are either present or accumulate in narcissus bulbs.

B. Phytoalexins

(i) Multicomponent response. Bioassay of Et_2O extracts of infected bulb tissue revealed the presence of induced antifungal compounds - phytoalexins. Eight were isolated and three fully characterised as closely related hydroxyflavan compounds (Fig. 3.2). This is only the fourth occasion that phytoalexins have been characterised from a monocotyledenous plant.

Narcissus bulbs produce 12 phytoalexins. Some early reports suggested that different plant species each produce a single, characteristic compound (Perrin and Bottomley, 1962; Perrin, 1964; Letcher, Widdowson, Deverall and Mansfield, 1970; Smith, McInnes, Higgins and Millar, 1971). Recently, Ingham (1977b) has found that Meliotus alba accumulates only one phytoalexin. However, it would now appear that this is an exception and multiple phytoalexin accumulation, often involving structurally related compounds, is the more common response to infection. Phaseolus vulgaris, for example, produces nine isoflavonoid compounds (Keen and Bruegger, 1977) and Vicia faba accumulates seven furanoacetylenic phytoalexins (Mansfield et al., 1980).

The phytoalexins from narcissus bulbs comprise two or more chemical families. Cowpea, broad bean, Trifolium spp. and vine also accumulate antifungal compounds of more than one structural class (Preston, Chamberlain and Skipp, 1975; Hargreaves et al., 1977; Ingham, 1978; Langcake, 1980).

The evolutionary processes which have resulted in a given species of plant producing several phytoalexins of diverse structure are open to speculation. Palaebotanical evidence suggests that plants have co-existed with fungal parasites for over 400 million years (Swain, 1978). Diversity of phytoalexins may be explained by a long period of coevolution; perhaps as strains of a fungus evolved that were able to detoxify one phytoalexin, only those individual plants survived that, fortuitously, produced a modified structure. By the same argument, there would be further selection of those plants able to produce phytoalexins in more than one chemical family - an ability that would

presumably confer on them a degree of resistance to a wider spectrum of pathogens. Whatever the evolutionary explanation, it would seem to be more difficult for a fungus to succeed against a plant capable of producing numerous antifungal compounds than against a plant producing only one.

(ii) Role in resistance. The apparent absence of phytoalexins in or at the edge of spreading lesions caused by B. narcissicola, and their rapid accumulation in limited lesions caused by B. cinerea, implicate these compounds as determinants of the outcome of Botrytis/narcissus interactions. As with cell wall alterations, convincing evidence for a role in resistance depends on showing that phytoalexins are in the right place at the right time and are present in sufficient quantity to inhibit growth of infection hyphae.

No quantitative measurements of the accumulation of individual phytoalexins were made in the present study. It should be possible however, using HPLC, to monitor accurately hydroxyflavan accumulation in narcissus. The accumulation of furanoacetylenic phytoalexins in Vicia faba has recently been thoroughly described using this method (Mansfield et al., 1980). In order to describe completely the antifungal environment in narcissus, the accumulation of uncharacterised phytoalexins, particularly PA1-4, also needs to be investigated on a quantitative basis.

A more difficult task in evaluating the role of phytoalexins in resistance is to determine the concentrations accumulating around infection hyphae. No-one has measured intracellular concentrations directly and the levels to which infection hyphae are exposed can only be estimated.

Several investigators have attempted to determine if estimated *in vivo* phytoalexin levels are sufficient to account for disease resistance; their work includes studies on rishitin in potato (Sato and Tomiyama, 1969), phaseollin in *Phaseolus vulgaris* (Bailey and Deverall, 1971), furanoacetylenic compounds in *Vicia faba* (Hargreaves *et al.*, 1977), medicarpin and isoflavan in lucerne (Flood, Khan and Milton, 1978) and glyceollin in soybean (Yoshikawa, Yamauchi and Masago, 1978). Recently, UV-fluorescence microscopy has localised more precisely the accumulation of wyerone acid in *Vicia faba* leaves (Mansfield, 1980) and the stress metabolites resveratrol and ε-viniferin in *Vitis vinefera* and *Vitis riparia* leaves (Langcake, 1980). More sophisticated forms of microscopy may eventually allow direct quantitation of phytoalexin accumulation in individual living cells and, more importantly, around invading hyphae.

It seems very probable that the accumulation of 12 phytoalexins within infected narcissus bulb tissue produces a highly fungitoxic environment. For example, five days after inoculation with *B. cinerea* conidia, an Et_2O extract at a concentration equivalent to 0.02g fr.wt. of inoculated tissue per ml was sufficient to inhibit the germ tube growth of *B. cinerea* sporelings in SPN by 50%.

The role of individual phytoalexins in the resistance of bulb scales to *Botrytis* can be assessed by comparing the concentration of each compound required to inhibit or kill *B. cinerea* sporelings *in vitro* (measured values) with the concentration accumulating *in vivo* (estimated values). Such comparisons depend on several assumptions:

1. Bioassay of phytoalexins in a nutrient solution at pH4

against pre-germinated spores accurately reflects the *in vivo* exposure of the fungus to an antifungal environment after infection (the pH of inoculum droplets fell to < 4 during infection).

2. The concentration of phytoalexins in bulb tissue 5 days after inoculation, the age of tissue from which milligram quantities of phytoalexins were isolated, are accumulated soon after infection and maintained at high levels.

3. All phytoalexins were completely extracted with Et_2O.

4. The density of bulb tissue is 1.5g/ml.

Accepting these assumptions, the contribution of each phytoalexin to the resistance of narcissus can be calculated as follows:

1. Individual phytoalexins have the following activities *in vitro* (Tables 3.20 and 3.21):

Phytoalexin	1	2	3	6	7	9
ED_{50} (mg/ml)	0.124	0.069	0.245	0.065	0.032	0.022
90% kill (mg/ml)	0.535	-	-	0.259	0.318	-

2. Amounts purified from selected gel filtration fractions were:

Phytoalexin	1	2	3	6	7	9
Amount (mg)	6.3	2.1	2.3	4.4	7.5	4.0

3. Estimated total amounts eluting from an extract of 60g fr.wt. of infected tissue:

Phytoalexin	1	2	3	6	7	9
Amount (mg)	12	4	5	22	38	24

4. Estimated phytoalexin concentrations *in vivo*, assuming 60g fr.wt. of infected tissue has a volume of $40cm^3$:

Phytoalexin	1	2	3	6	7	9
Concentration (mg/ml)	0.30	0.10	0.13	0.55	0.95	0.60

Comparing (1) and (4), five out of six phytoalexins appear to be present within tissues at concentrations sufficient to inhibit sporeling growth by at least 50% and two out of three phytoalexins are present at levels greater than those killing 90% of sporelings *in vitro*.

Two further points are worth considering. First, the concentration of phytoalexins at sites of attempted infection may in fact be considerably higher than indicated in (4). This could arise from (i) a non-uniform distribution of phytoalexins within cells and/or within limited lesion tissue or (ii), the inclusion of healthy tissue, not responding to infection, in limited lesion collections. Second, although phytoalexins may not be present initially at levels sufficient to kill or completely inhibit the growth of sporelings, they may still be present at levels which reduce the speed of infection development and perhaps permit restriction of infection by a second resistance mechanism (e.g. lignification).

The role of phytoalexins in disease resistance was recently discussed by Smith (1978) with reference both to whether or not such compounds are responsible for the failure of fungi to colonise tissue and also to their involvement in the determination of specificity of host-parasite interactions.

In future research into the role of phytoalexins in disease resistance, the use of mutations may prove useful. For example, it would be interesting to examine the virulence of isolates of a pathogenic fungus showing altered phytoalexin sensitivity. Investigation into the susceptibility of mutant plant lines, with differing capacities to accumulate

phytoalexins, might also provide evidence to help support or refute the hypothesis that phytoalexins are responsible for disease resistance.

(iii) Structure and fungitoxicity of flavonoids. The phytoalexins isolated from narcissus are hydroxyflavans. Flavonoid compounds unsubstituted in the pyran ring are rare natural products, having only been reported as minor constituents in the roots of Dianella revoluta and Stypandra grandis (Xanthorrhoeceae)(Cooke and Down, 1971) and in the resin from Xanthorrhea preissii (Birch and Salahuddin, 1964). Although at least 14 isoflavonoid phytoalexins have been characterised, this is the first report of flavans possessing antifungal activity and accumulating in response to infection. The only other flavonoid phytoalexin characterised is a very weakly antifungal flavanone, betagarin, isolated from Beta vulgaris (Geigert, Stermitz, Johnson, Maag and Johnson, 1973).

Without a standard bioassay technique it is difficult to compare the antifungal activities of different phytoalexins. However, the phytoalexins from narcissus can be compared with those from Vicia faba as all have been assayed against B. cinerea sporelings in SPN. Using this assay, 7-hydroxyflavan, the most active narcissus phytoalexin, is considerably less active than the major furanoacetylenic phytoalexins from V. faba (Table D.1).

Substituents in the flavan ring were found to be important in determining whether or not flavonoids were fungitoxic but an unambiguous relationship between structure and activity was not revealed. Thus, the majority of fungitoxic compounds had only one or two polar groups

TABLE D1 Comparison of the antifungal activity of phytoalexins from narcissus and Vicia faba by bioassay against sporelings of B. cinerea

Plant	Phytoalexin	ED_{50}[a] µg/ml	µM
Narcissus	7-Hydroxyflavan	22.1	97.7
	7,4'-Dihydroxyflavan	64.6	266.9
	7,4'-Dihydroxy-8-methylflavan	31.8	124.2
	PA1 (non-phenolic)	124.0	734
	PA2 "	68.8	346
	PA3 "	244.8	1149
Vicia faba	Wyerone	10.1	39.1
	Wyerone acid	3.5	14.3
	Wyerone epoxide	2.7	23.4
	Wyerol	85.0	32.6
	Wyerol epoxide	c.500	c.1811
	Dihydrowyerol	>1000	>3800
	Reduced wyerone acid	>1000	>4000
	Medicarpin	14.0	51.8

a Concentration of phytoalexin in SPN at pH4 which redcued germ tube growth by 50%; determined from graphs of germ tube growth against phytoalexin concentration. Values for V. faba phytoalexins are taken from Hargreaves, Mansfield and Rossall (1977) and Rossall (1978).

(usually including a C-7 hydroxyl) and those with three or four were inactive; methylation of hydroxyl groups appeared to eliminate antifungal activity. Similar observations on the number and polarity of substituents were made by Carter, Chamberlain and Wain (1978) in a study of 24 hydroxyl and methoxy derivatives of the phytoalexin vignafuran (2,6,2'-trimethoxy-4'-hydroxybenzofuran) and by Ingham (1977a), who noted an inverse relation between the number of hydroxy groups on compounds related to demethylvestitol, a trihydroxyisoflavan phytoalexin, and antifungal activity. The latter worker ascribed increasing antifungal activity to increasing methylation of hydroxyl groups but did not report on the trimethoxyisoflavan.

For isoflavonoid phytoalexins with a pterocarpan structure, Perrin and Cruickshank (1969) proposed that a common molecular shape with small O-containing substituents at C-3 and C-9 was responsible for activity. They further suggested that sensitive fungi had a structure-specific receptor, probably located in a membrane. Activity in the isoflavan analogues of 7-hydroxyflavan and 7,4'-dihydroxyflavan would seem to preclude the possibility that a common molecular configuration is responsible for activity of all flavanoids. In further studies on the pterocarpans however, Van Etten (1976) provided convincing evidence to refute the hypothesis of Perrin and Cruickshank that antifungal activity depends on a common three-dimensional shape.

Thus, for both flavonoids and isoflavonoids the evidence appears to be more in favour of the hypothesis that antifungal activity depends on some common physicochemical attribute, perhaps lipophilicity and an ability to penetrate

fungal membranes, rather than a common structure. The alternative explanation cannot be excluded as different compounds may have different modes of action. For example, if several receptor sites exist in the fungal cell, three dimensional structure may still be an important feature for activity but a common structure need not exist. Studies on the mode of action of flavonoid phytoalexins should help to elucidate the basis of apparent structural relationships.

The strictly-specific antibacterial activity of hydroxyflavan and other phytoalexins towards Gram-positive types (Gnanamanickam and Smith, 1980; Gnanamanickam and Mansfield, 1980), does not clarify the basis of antimicrobial activity. The well-documented differences in wall structure between Gram-negative and Gram-positive bacteria and the lipophilic nature of many antibacterial compounds suggest that activity may depend on an ability to enter cells. However, it could also be that only Gram-positive bacteria possess specific receptors in membrances to which active compounds bind.

4. General discussion

A. Pathogenicity of B. narcissicola

If phytoalexins and cell wall alterations are the mechanisms responsible for resistance in narcissus bulbs, the ability of B. narcissicola to overcome these mechanisms needs to be explained.

Tolerance to phytoalexins does not appear to be an explanation for the virulence of B. narcissicola as the sensitivity of B. narcissicola sporelings was similar to

that of B. cinerea and non-pathogenic Botrytis spp. This is in contrast to the differential tolerance of Botrytis spp. to the furanoacetylenic phytoalexins from V. faba (Hargreaves et al., 1977) or to medicarpin and maackiain from clover (Macfoy and Smith, 1979). In both these interactions the pathogenic species were the least sensitive to the host phytoalexins and also had the greater capacities to metabolise their host's phytoalexins. Nuesch (1963) suggested that Rhizoctonia solani is able to rot bulbs of Orchis militaris as a result of its capacity to degrade the orchid phytoalexin, orchinol. The ability of B. narcissicola and B. cinerea to metabolise narcissus phytoalexins was not investigated.

The striking feature about spreading lesions caused by B. narcissicola was the absence of significant levels of phytoalexins and the deposition of reaction material. This indicates a suppression of, or a failure to elicit, the host's mechanisms of active resistance. Probably the simplest explanation is that B. narcissicola is specifically adapted to narcissus and, given a high inoculum potential, can rapidly invade tissue causing a non-specific suppression of active resistance by killing host cells. The ability of B. narcissicola to cause spreading lesions from conidial inocula when the inoculum potential is raised by adding nutrients or wounding tissue, treatments which lead to increased rates of infection development, supports this hypothesis.

Phytoalexins were also notable by their absence from bulb tissue inoculated with mycelium of B. fabae and B. tulipae. The failure to detect phytoalexins here appears unlikely to result from a suppression of resistance by cell killing. Most

probably it results from a failure to elicit phytoalexin production. Neither species grew well over narcissus tissue and fleck lesions were absent or rare. By contrast, mycelial inocula of B. cinerea, inocula which consistently led to the formation of discrete limited lesions, elicited a marked phytoalexin accumulation.

An important factor in determining the outcome of host-parasite interactions in the relative speed of infection development by the fungus and active response by the host. A specific ability to kill a large number of host cells very soon after penetration may be a common attribute of host-specific Botrytis species (Mansfield, 1980); phytotoxins may therefore be an important factor in determining specificity. In this context, Harrison (1980) has recently shown that phytotoxins extracted from diseased bean leaves were specific in their activity towards leaves of Vicia faba, failing to cause lesions when injected into 13 other plant species.

B. Interdependence and control of resistance mechanisms

Two mechanisms of active resistance, apparently quite distinct, have been demonstrated in narcissus bulbs. On closer examination however, it is seen that these two mechanisms are connected by a common requirement in the initial stages of their biosynthetic pathways. Both pathways have a requirement for phenylpropanoid phenolic acids.

To meet an increased demand for phenolic acids following infection, an increase in the activity of PAL would not be unexpected. Dixon and Bendall (1978a,b) using cell suspension cultures of Phaseolus vulgaris, elicited a specific stimulation of the synthesis of both isoflavonoids

(including the phytoalexin phaseollin) and wall-bound phenolics which correlated with significant increases in the extractable activities of PAL, flavanone synthase and other enzymes of phenylpropanoid metabolism. PAL increases correlated best with the appearance of wall-bound phenolics.

The pathways of lignin and flavonoid synthesis diverge after p-coumaryl-coA and similar coA esters of other phenolic acids. Entry of these esters into flavonoid synthesis is controlled by flavanone synthase and entry into lignin synthesis by reductase enzymes (Hahlbrock and Grisebach, 1979). It would be interesting to examine whether these and other enzymes, at later stages in the pathways of lignin and flavonoid phytoalexin synthesis in narcissus, show correlated (de novo) increases in activity following attempted infection. If so, this would be evidence for co-ordination of resistance mechanisms, perhaps the result of a common elicitor.

The operation in parallel of more than one mechanism of active resistance would appear to be an effective means of resisting invasion. For example, an initial cell wall alteration might delay infection development sufficiently to allow the accumulation of phytoalexins to fungitoxic levels. Alternatively, phytoalexins might sufficiently inhibit fungal growth following infection to permit the development of a cell wall barrier, preventing further spread. Resistance in practice probably lies somewhere between these extremes and, where two or more mechanisms are present, their co-ordinated operation may determine the outcome of a host parasite interaction.

The potential importance of co-ordinated multiple mechanisms of resistance was recently stressed by Kuc and Caruso (1977) and Mansfield (1980). Langcake and Wickins (1975a,b) and Cartwright et al. (1977) have provided evidence that both a melanoid pigment and phytoalexins are involved in the resistance of rice leaves to Piricularia oryzae. Sakuma et al. (1976) showed that oxidised phenols, as well as phytoalexins, have a role in the resistance of red clover to infection by Kabatiella caulivora. Mace (1978) found that both tyloses and terpenoid phytoalexins contribute to Verticillium wilt resistance in cotton. The demonstration of both phytoalexin accumulation and lignification in narcissus lends further support to the hypothesis that resistance results from the operation of multiple mechanisms.

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APPENDIX 1

BULB STRUCTURE

The narcissus bulb is a complex structure comprised of fleshy scales, leaf bases and inflorescences. The components may be envisaged as arranged into bulb 'units' of different generations (Rees, 1969). From the outside inward each bulb unit has scales (2-4), leaf bases (usually three) and an inflorescence. Within this unit are terminal and lateral buds, bulb units of the next generation (Fig. Ala). As the younger bulb units grow and develop, the surrounding scales, leaf bases and flattened inflorescence bases of older units are gradually pushed towards the outside of the bulb. By the time they have reached the outside of the bulb tissues have become brown and papery-thin. A typical 'double-nose' bulb (Fig. Alc) may contain three generations of complete bulb units and part of a fourth, oldest generation, on the outside. Each bulb unit thus lives for about 4 yrs. Routine pathogenicity tests were carried out on a selection of fleshy bulbs scales and leaf bases enclosing terminal or lateral buds, second generation tissue.

FIG. A1 Diagrams of narcissus bulbs of increasing age and complexity in transverse section; (a) the basic bulb unit (tissues drawn expanded); (b) a 'large round'; (c), a 'double nose' and (d), a 'mother bulb'. Adapted from Anon. (1964) and Rees (1969).

Key:		
	FBd	Flower bud of the coming season
	TBd	Terminal bud (future bulb unit)
	LBd	Lateral bud (" " ")
	L	Foliage leaf
	LB	Leaf base
	S	Bulb scale
	OFS	Old flower stalk base
	MS	Membranous scales (not distinguishable as scales or leaf bases)

Numbers (1,2,3) refer to similar parts in consecutive years and letters (a,b,c) to similar parts in the same year.

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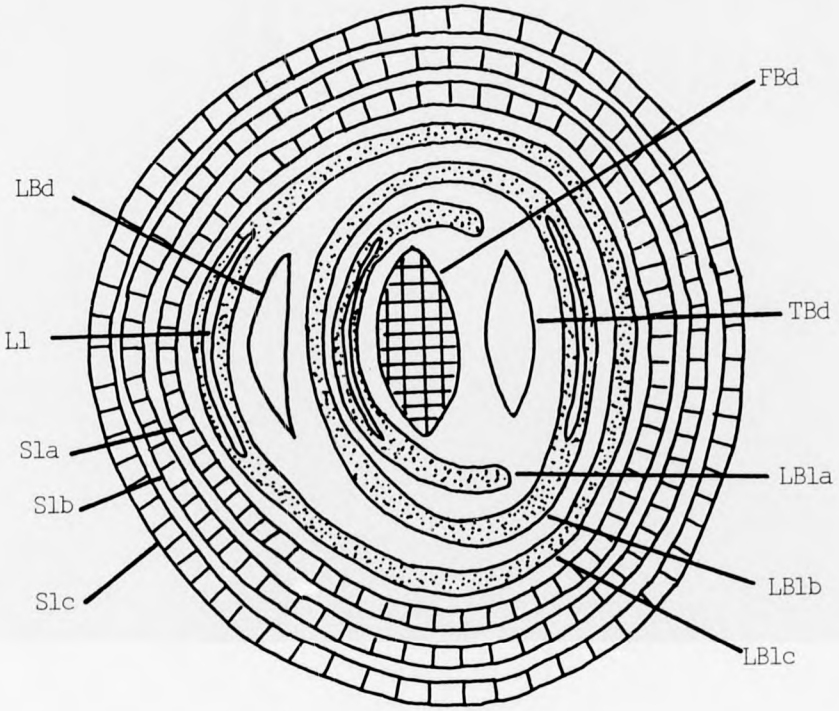
a



b

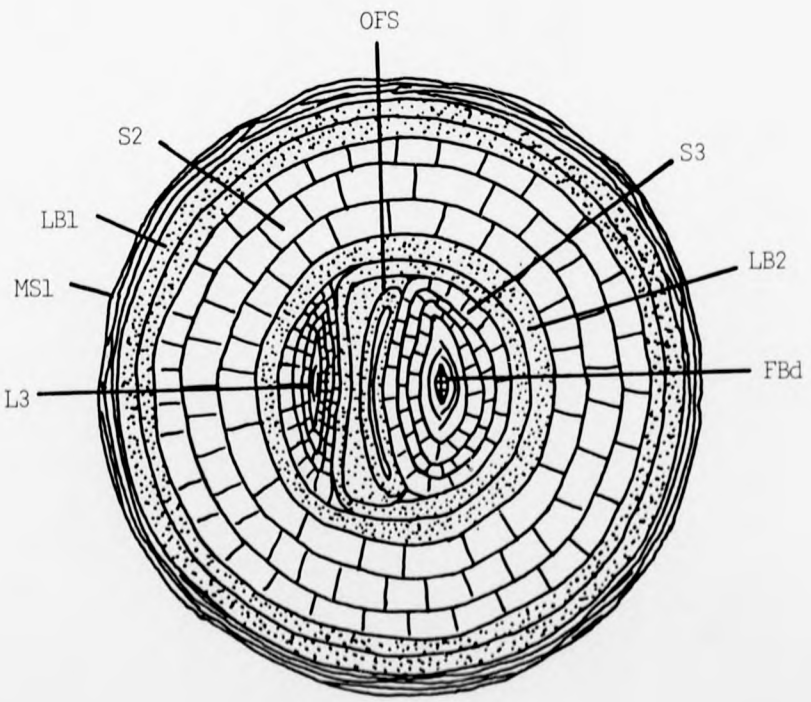
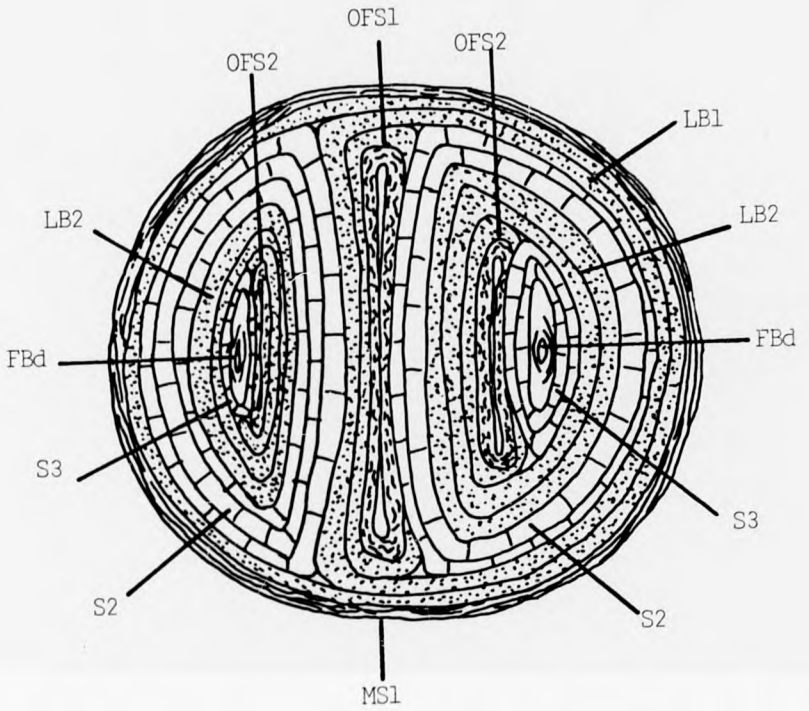


FIG. A1

c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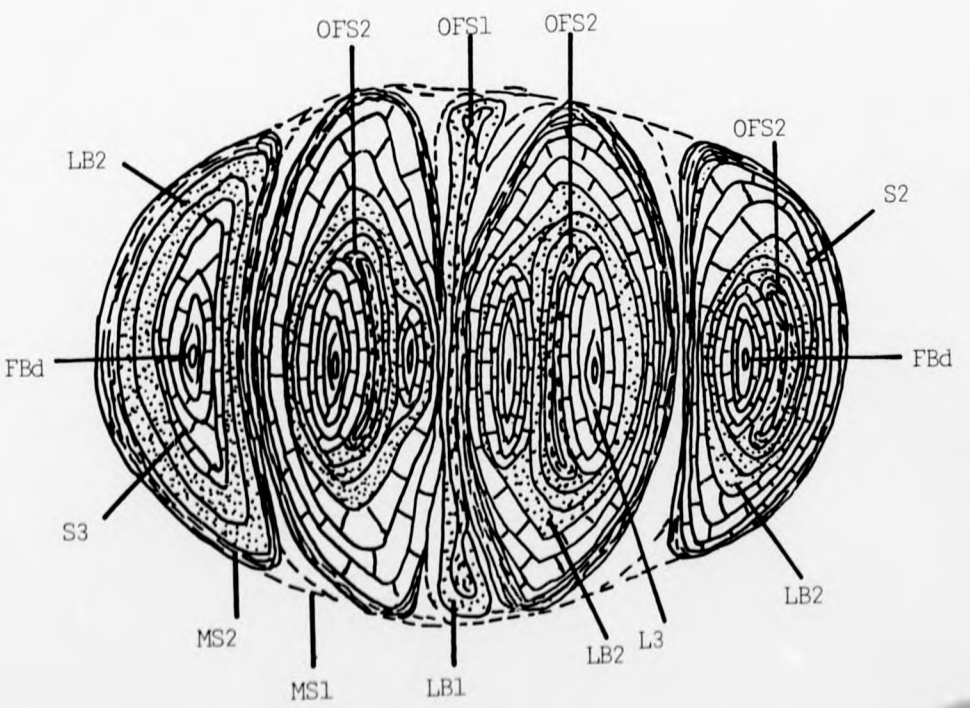
OFS2
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OFS2
OFS2
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FBd
S3
LB2
MS2
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L3
MS1
LB1

FIG. A1

APPENDIX 2

UV ABSORBANCE BY HYDROXYFLAVAN PHYTOALEXINS IN SPN

When determined by UV absorbance, the concentration of hydroxyflavan phytoalexins in SPN bioassay test solutions, prepared from methanolic solutions of known concentration, was found to be less than that predicted by calculation. The λ_{max} UV absorbances of these compounds in MeOH and in SPB/2% DMSO, were therefore compared.

The λ_{max} UV absorbance of 7-hydroxyflavan (PA9) was found to be identical in SPN and MeOH but the absorbance of 7,4'-dihydroxyflavan (PA6) and 7,4'-dihydroxy-8-methylflavan (PA7) were 7.35 and 11.85% lower in SPN than in MeOH (Table A1). Correction factors of 1.08 (PA6) and 1.13 (PA7) are therefore required when the concentration of these two phytoalexins in aqueous solutions are determined by UV absorbance.

TABLE A1 UV absorbance by hydroxyflavan phytoalexins in MeOH and in SPN/DMSO

Experiment	Phytoalexin	Absorbance at λ_{max}. MeOH	SPN/DMSO	% low in SPN
1	7,-Hydroxyflavan	0.088	0.088	0
	7,4'-Dihydroxyflavan	0.136	0.126	7.3
	7,4'-Dihydroxy-8-methylflavan	0.245	0.205	16.3
2	7,-Hydroxyflavan	0.530	0.530	0
	7,4'-Dihydroxyflavan	0.810	0.750	7.4
	7,4'-Dihydroxy-8-methylflavan	0.605	0.560	7.4

APPENDIX 3

UV ABSORBANCE BY METHANOLIC SOLUTIONS OF FLAVONOID COMPOUNDS

The UV absorbance extinction coefficients used to calculate the concentration of flavonoid compounds assayed for antifungal activity are given in Table A2.

TABLE A2 UV absorbance by methanolic solutions of flavonoid compounds assayed for antifungal activity

Compound	Mol.wt.	λ_{max} (nm)	ε	Conc. (μM) at 1 a.u.	Reference
Flavone	222	294	25,789	39	Láng (1966-1969)
Flavanone	224	252	8,800	114	Láng (1966-1969)
Liquiritigenin	254	277	13,800	72.5	Ingham (Pers. comm.)
Naringenin	272	290	16,366	61	Láng (1966-1969)
7-Methoxy-4'-hydroxy-8-methylflavan	256	281	4,677	214	Cooke and Down (1971)
7-Hydroxyflavan	226	285	3,070	326	Coxon et.al., (1980)
7,4'-Dihydroxyflavan	242	284	4,680	214	Coxon et.al., (1980)
7,4'-Dihydroxy-8-methylflavan	256	279	4,020	249	Coxon et.al., (1980)

II

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END

82