Project Title:	Biology and control of clematis wilt
Project Number:	HNS 52
Report:	Final Report
Earlier Reports:	Annual Report Year 1 (Sept. 1997) Annual Report Year 2 (Oct. 1998)

Project Leaders:	Dr T M O'Neill ADAS Arthur Rickwood Mepal, Ely Cambs. CB6 2BA Dr J M Chartier-Hollis University of Derby School of Environmental and Applied Sciences Kedleston Road Derby DE22 1GB
Key Staff:	P van de Graaf (researcher and author) Dr M E Joseph, University of Derby (supervisor)

Location:	University of Derby and ADAS Arthur Rickwood

Project Co-ordinators:	R N Pennell Pennells Nurseries Newark Road South Hykeham Lincoln LN6 9NT	H D W Baird Corachie Clematis Taynuilt Argyll Scotland PA35 1HY

Date Project Commenced:	1.10.1996
Date Project Completed:	1.10.1999
Key words:	clematis, wilt, leaf spot, stem rot, root rot, *Phoma clematidina*, survey, resistance, control, prevention, spore germination, mycelial growth, host-pathogen interactions, fungicide, spread, watering method

Whilst reports issued under the auspices of the HDC are prepared from the best available information, neither the authors or the HDC can accept any responsibility for inaccuracy or liability for loss, damage or injury from the application of any concept or procedure discussed.

© 1999 Horticultural Development Council
No part of this publication may be reproduced in any form or by any means without prior permission from the HDC.

PRACTICAL SECTION FOR GROWERS

Aims of the project

The aims of this project were to study the biology and causes of the main disease of clematis, 'clematis wilt', and to optimise disease prevention and control. In addition, the possibility of developing a simple test to screen clematis plants for susceptibility to wilt was investigated.

Summary of results

Postal surveys of commercial and amateur clematis growers showed that clematis wilt was widespread and common both on nurseries (50% in 1991-1996) and in private gardens (81%) throughout the UK. A clear difference in susceptibility between large (susceptible) and small (resistant) flowered clematis was reported. Many commercial growers experienced problems with chemical control of the disease and reported annual losses up to 30%. Visits to several nurseries in both England and the Netherlands, from where many clematis plants are imported, confirmed the widespread occurrence of clematis wilt. Fungal isolations from diseased plants from British nurseries and private gardens yielded a wide range of fungal species. *Phoma clematidina* was most commonly isolated and detected in 23% of plants. This fungus was found to be able to cause leaf spot, stem rot and wilt in healthy clematis plants, while none of the other fungi tested, including *Coniothyrium*, *Fusarium* and *Botrytis*, caused wilting symptoms. *Phoma clematidina* was also found to be able to infect clematis roots and cause severe root rot, which had not been reported previously and which means that the practice of cutting back wilted plants to grow them on is not a reliable method to remove infection.

Studies were undertaken to investigate fungal growth on different agar media. Vegetable juice (V8) agar was found to be most suitable for *P. clematidina*. Chlamydospores were formed by all isolates of the fungus and provided a useful stable characteristic for identification. The fungal isolates could be divided into three morphological groups, differing in the number of pycnidia, spore mass, mycelium and chlamydospores formed. Tests with agar amended with fungicides showed that these groups were correlated with resistance to benzimidazole fungicides. One third of the isolates of *P. clematidina* recovered were found to show resistance to benomyl (Benlate), carbendazim (Bavistin DF) and thiophanate-methyl (Mildothane Liquid). Benzimidazole resistant strains of the fungus were less virulent (produced less severe symptoms) on healthy clematis leaves than were sensitive isolates.

Both mycelial growth and spore germination of *P. clematidina in vitro* were optimal at a temperature around 20 °C, but also took place at much lower temperatures. Germination of spores occurred in pure water within 6 hours after suspension and single celled spores formed up to four germ tubes each. The addition of nutrients to the water promoted spore germination and germ tube growth. Using light microscopy and Scanning Electron Microscopy (SEM), it was found that *P. clematidina* penetrated epidermal cells of clematis directly, with or without the formation of special infection structures. The fungus completely ignored the stomata as points of entry, but regularly entered the plant by penetrating leaf hairs.

©1999 Horticultural Development Council

More germ tubes were formed by spores on the plant surface of the wilt susceptible clematis variety tested (*C.* 'Henryi') than on the resistant variety (*C. montana*). However, the main difference between the two varieties was that only on the resistant variety, were haloes formed in the plant epidermis in reaction to *P. clematidina*, preventing the fungus from further invasion and regularly resulting in subcuticular germ tube growth. This indicated that resistance mechanisms of small flowered clematis to disease caused by *P. clematidina* were activated even before fungal invasion of any plant tissues took place. The difference in susceptibility between the two varieties also became apparent by inoculation of unwounded leaves and stems with *P. clematidina*. Lesions on *C. montana* remained limited in contrast to those on *C.* 'Henryi' which spread quickly. Inoculation of internodal stems was much more destructive than inoculation of leaves and led to wilt of all susceptible plants within 30 days. Therefore, this method was used to test 11 different clematis varieties on their susceptibility to infection by *P. clematidina*. A positive linear relationship was found between the susceptibility score in the stem inoculation trial and the score for susceptibility to wilt in practice given by growers in the 1996 survey. This means that a stem inoculation test with *P. clematidina* might be useful for predicting wilt susceptibility of new clematis varieties. Only for *C. viticella* and related varieties would this the stem inoculation test be less accurate, since they are less susceptible to wilt in practice than to stem infection by *P. clematidina* in inoculation tests.

P. clematidina was found to be capable of surviving, growing and reproducing on dead plant material, not only of clematis but also of some common weeds. This means that nursery hygiene should take an important place in prevention of clematis wilt.

Since benzimidazole fungicides were found to be ineffective against some strains of *P. clematidina*, a range of other fungicides were tested on their effect on mycelial growth and spore germination of this fungus in the laboratory. Most effective were difenoconazole (Plover), azoxystrobin (Amistar), kresoxim-methyl (Stroby) and pyrimethanil (Scala) and therefore these fungicides, together with carbendazim (Bavistin DF) and prochloraz (Octave), were tested on clematis plants inoculated with spores of *P. clematidina*. Pyrimethanil was least effective and in addition had phytotoxic effects. However, difenoconazole and the two strobilurins were very effective in reducing disease and might form good alternatives to benzimidazole fungicides for the control of clematis wilt.

Two different watering methods were tested to compare their effect on spread of disease by *P. clematidina* within groups of healthy clematis plants. Disease spread quickly within groups which were watered overhead, because the fungal spores were distributed by water splash and moisture was created on the plant surface which enabled spore germination and infection. However, in groups which were drip irrigated, disease incidence was very low and disease spread limited. The results showed that with drip irrigation, fungicide treatment (Octave and Bavistin DF) was unnecessary in contrast to the situation with overhead watering.

©1999 Horticultural Development Council

Action points for growers

- General nursery hygiene is essential in the prevention of clematis wilt. Dead plant material of clematis and other plants should be collected and destroyed on a regular basis. Leaves with spots should also be removed when found. Pruning tools should be disinfected between batches of plants.

- Wilted plants and those which show no fresh growth in spring may be infected with *P. clematidina* and could form a source of infection for healthy plants nearby. Such plants should be removed and destroyed as soon as detected.

- The use of benzimidazole fungicides, especially carbendazim (Bavistin DF), will be ineffective against *P. clematidina* if resistant strains of the fungus are present. Difenoconazole (Plover), azoxystrobin (Amistar) and kresoxim-methyl (Stroby) are better alternatives but not allowed for use on clematis as yet. It is best to alternate several fungicides with different modes of action, on a regular basis, to prevent selecting resistant strains.

- Moist conditions are favourable to the spread of and infection by *P. clematidina,* and to the development of rot and leaf spot symptoms. Leaves, stems and roots of clematis should not be subject to long periods of constant wetness as may occur following overhead watering. Watering plants by drip irrigation, or another method which does not wet the above ground parts of the plants, can significantly reduce the incidence of clematis wilt and may make regular fungicide treatments against clematis wilt unnecessary.

Practical and anticipated financial benefits

The results of the 1996 survey clearly showed that the losses due to clematis wilt per annum in the UK have been considerable. Furthermore, the sale of plants might have been tempered by the negative image of the crop with the general public because of wilt susceptibility. The findings of this project led to a much better understanding of the biology of the disease and showed that cultural practice and chemical control of clematis wilt can be significantly improved. Implementation of this knowledge in practice in the form of an integrated control strategy should eventually lead to a substantial reduction in losses and a better quality of the crop in general.

©1999 Horticultural Development Council

CONTENTS

Practical section for growers 1

Science section

1. Introduction 4

2. Grower surveys 7

3. Nursery visits and fungal isolations 8

4. Growth of *Phoma clematidina* on agar 9

5. Spore germination and infection studies with *Phoma clematidina* 13

5.1 Effect of different factors on spore germination in water 13
5.2 Studies of spore germination on leaves at light microscopy level 16
5.3 Studies of spore germination and infection at SEM level 17

6. Disease development studies with *Phoma clematidina* 25

6.1 Virulence of different fungal isolates 25
6.2 Disease development on leaves and stems of two varieties 25
6.3 Disease development on roots 27
6.4 Disease development in a range of varieties 28

7. Chemical control and cultural factors 34

7.1 Benzimidazole resistance *in vitro* 34
7.2 Evaluation of fungicides *in vitro* 36
7.3 Evaluation of fungicides *in vivo* 39
7.4 Effect of watering method on disease spread and control 43
7.5 Effect of growing medium on root infection 47
7.6 Saprophytic growth on plant material 48

8. Overall discussion 50

9. Conclusions 53

References 54

Acknowledgements 56

© 1999 Horticultural Development Council

SCIENCE SECTION
1. INTRODUCTION

Aims of the project

The aims of this project were to study the epidemiology and causal organism(s) of the main disease of clematis, 'clematis wilt', and to optimise disease prevention and control. In addition, the possibility of developing a simple test to screen clematis plants for susceptibility to the disease was investigated.

Clematis wilt

The crossing of newly discovered clematis species from the Far East with European species in the 18th and 19th Century led to large flowered hybrids which became very popular around the world. However, soon after their large scale introduction, reports began to appear in both American and European journals about a widespread, destructive disease which caused huge losses, discouraged the breeding of new varieties and eventually forced many growers to discontinue their clematis production. The symptoms of this disorder, which became known as 'clematis wilt', were usually described as stem lesions (mostly at soil level) and consequently wilt of all above ground parts of the plant (Arthur, 1885; Comstock, 1890; Foussat, 1896; Sorauer, 1897; Jackman, 1900). Early speculations about the main causal organism of clematis wilt included fungi, bacteria, nematodes and insects (Van de Graaf, 1997), while purely physical factors have also been blamed (Howells, 1993) .

The fungus *Phoma clematidina*

Clematis wilt was first associated with a fungus, a *Phoma* species, by Arthur in 1885, after which fungi were regularly noticed, but never thoroughly investigated (Foussat, 1896; Sorauer, 1897). However, significant research into the fungal causes of clematis wilt was eventually published by the American botanist Gloyer (1915), who established Koch's postulates for *Phoma clematidina* (Thüm.) Boerema, formerly known as *Ascochyta clematidina*, as a cause of leaf spot, stem rot and wilt in clematis. This fungus has now been recognised as the most important cause of clematis wilt by scientists around the world (Ebben & Last, 1966; Smith & Cole, 1991; Van Kuik & Brachter, 1997).

P. clematidina can easily survive as a saprophyte on dead material of different plant species, but as a pathogen seems to be specific to clematis. Both Gloyer (1915) and Spiers (1994) tested its pathogenicity towards a range of other plants, but no significant symptoms were formed even on plants closely related to clematis such as anemone, buttercup and delphinium. On the basis of data from the literature (Boerema, 1993) and observations in the current project, a life cycle of the fungus was compiled (see Figure 1). The fungus reproduces by forming asexual spores (conidia) in spherical fruiting bodies known as pycnidia. The spores are produced in a salmon coloured mass and mainly spread by water droplets (rain, overhead watering). The germination process also takes place under moist conditions and may include conidial swelling and septation.

©1999 Horticultural Development Council

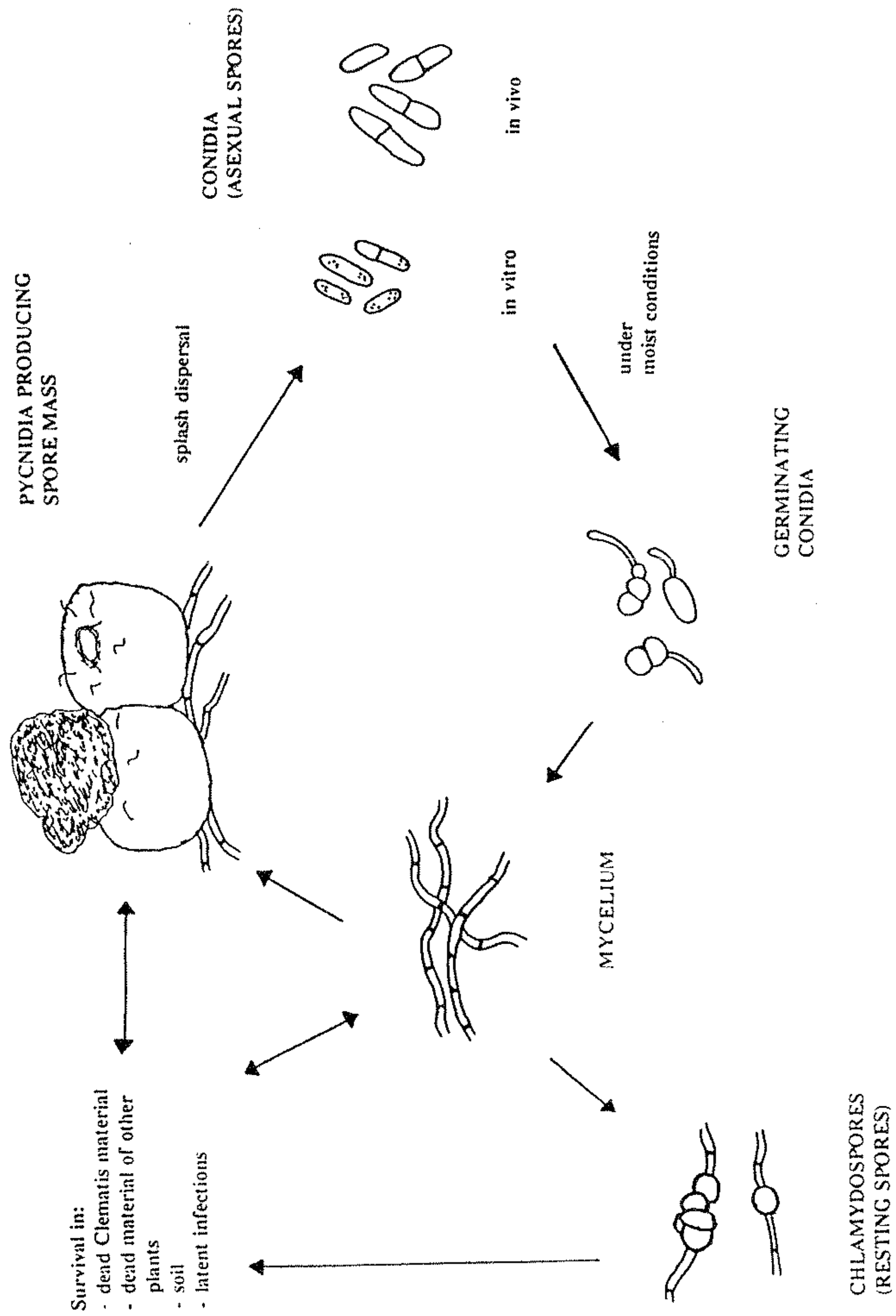


Figure 1. Life cycle of *Phoma clematidina* on cultivated clematis plants.

Conidia formed on plant material (*in vivo*) can be two-celled and larger than those produced on artificial media (*in vitro*), but this probably depends on environmental factors (Boerema *et al.*, 1997). Thick walled resting spores (chlamydospores) are usually formed in the fungal mycelium and their shape is important to distinguish *P. clematidina* from other *Phoma* species occurring on clematis. The geographical origin of *P. clematidina* is unknown, but the species is now widespread around the world (Boerema, 1993). This could be the result of the world-wide trade in clematis, but it is also possible that the fungus originates from wild clematis species, since Spiers (1991) found it very regularly on *C. lingusticifolia* and *C. vitalba* collected from the wild throughout the USA, UK and Europe. Two of the isolates Spiers discovered differed from the regular *P. clematidina* strains in that they formed relatively large two-celled conidia *in vitro* instead of the normal, smaller one-celled spores. More importantly they also formed a sexual stage (teleomorph) *in vitro*, which had not been reported for this fungus before. This new discovery is still under investigation (Spiers, pers.comm.) and since the sexual stage of *P. clematidina* has never been found on cultivated clematis and is possibly only formed by strains of the fungus which are specialised on wild, small flowered species (De Gruyter, pers.comm.), the sexual stage is not included in the life cycle in Figure 1.

Susceptibility to clematis wilt

Ever since clematis wilt first became a problem in the 19th Century, it has been clear that there are differences in susceptibility to this disease between different clematis varieties and species. The large flowered hybrids seem to be especially prone to stem rot and wilt, while most small flowered species and their varieties are less susceptible (Foussat, 1896). Within the large flowered varieties, it is especially the plants in the early flowering group that often succumb to wilt (Howells, 1994a). The basis of the resistance mechanism in small flowered clematis is not fully understood, although a difference in susceptibility to a toxin formed by *P. clematidina* may play a role (Smith, 1987; Smith *et al.*, 1994).

Control methods

Protective fungicides were applied against clematis wilt even before *P. clematidina* was identified as the causal organism. The famous Bordeaux mixture was tried, but with varied results (Anonymous, 1898; Gloyer, 1915). The systemic fungicide benomyl (Benlate) has long been the chemical most widely used against the disease and has showed to be fairly effective on nurseries as well as in private gardens. In trials with *P. clematidina,* it often proved to be the best fungicide tested (Jones, 1981; Wolff, 1996). Carbendazim (Bavistin DF) and thiophanate-methyl (Mildothane liquid), which have the same mode of action as benomyl, are generally thought to be somewhat less effective. As a result of the widespread use of these benzimidazole fungicides, it was discovered in 1996 that some Dutch isolates of *P. clematidina* had developed resistance against them (Van Kuik & Brachter, 1997). Several researchers foresaw the development of benzimidazole resistance and tested a range of fungicides on *P. clematidina* in the search for good alternatives (Jones, 1981; Smith & Cole, 1991; Wolff, 1996). However, no fungicide was found that matched the effectiveness of benomyl. Regardless of fungicide regime, strict general hygiene forms an important factor in the prevention of clematis wilt on nurseries (Gloyer, 1915).

©1999 Horticultural Development Council

2. GROWER SURVEYS

Aim

To establish the incidence of clematis wilt on nurseries and in private gardens throughout the UK and identify factors possibly related to disease occurrence.

Methods

Two postal surveys, one for commercial growers and one for amateur growers, were sent out in 1996. The number of respondents was respectively 76 and 158.

Main results & Discussion

The occurrence of clematis wilt in the period 1991-1996 was reported by 50% of commercial growers and 81% of amateur growers. Estimated losses on clematis nurseries were mostly around 5%, but much higher losses up to 30% were occasionally indicated. Management of clematis wilt consisted mostly of removal of dead plant material (50%), application of fungicides (63%) and removal of wilted plants from production (60%). However, 29% of commercial growers with problems also cut back diseased plants in the hope of re-growth. This control method does not guarantee the elimination of *P. clematidina* which can also be present on roots (see Sections 3 & 6). Different fungicides were usually alternated and applied on a fortnightly basis. Most popular were Benlate (benomyl), Octave (prochloraz) and Rovral (iprodione). Of the 37% of growers with wilted plants who did not apply fungicides at all, many wrote that they believed that these chemicals had lost their effectiveness against the disease. The development of resistance against benzimidazole fungicides in some strains of *P. clematidina* may partially explain this (see Section 7). In case of the amateur grower survey, no factors which had generally been assumed to play an important role in disease development such as full sunlight, unshaded roots, moist weather, irregular watering, bad plant hygiene and damage to the plant could be associated with the occurrence of wilt. However, 60% of respondents with problems did not apply fungicides and 85% had experienced trouble with clematis wilt in their gardens before. Also, several amateur growers commented on the occurrence of wilt after strong winds due to mechanical damage.

In both the commercial and amateur grower survey, respondents ranked small flowered clematis as very resistant, although there were some exceptions such as *C. florida* and *C.* x *triternata* 'Rubro-marginata', which according to amateur growers are more susceptible to wilt. Both surveys also largely agreed on the susceptibility of the large flowered varieties. *C.* 'Vyvyan Pennell', *C.* 'Henryi', *C.* 'Marie Boisselot' and *C.* 'Mrs N Thompson' were judged as very susceptible while *C.* 'Hagley Hybrid' and *C.* 'Ville de Lyon' were indicated as the most resistant large flowered varieties. These results agree with those reported by Howells (1994b). Thus, the surveys confirmed that the variety of clematis grown is a major factor in the occurrence of clematis wilt in practice.

Full results of the survey are given in the First Annual Report (September 1997).

©1999 Horticultural Development Council

3. NURSERY VISITS AND FUNGAL ISOLATIONS

Aim

To investigate growing methods used by clematis growers which may affect disease occurrence, to identify fungi present in diseased plant material, and to test possible pathogens found for pathogenicity towards clematis.

Methods

Six different British and Dutch clematis nurseries were visited in the period 1996-1997. Diseased clematis plants were collected during nursery visits in the UK or sent in by mail from commercial and amateur growers. Pieces of diseased leaf, stem or root were disinfected and plated out on V8-agar with added antibiotics. Fungal colonies were identified microscopically with the aid of descriptions in the literature. *Fusarium* species were identified by the Central Science Laboratory (CSL), some *Phoma* species by the Dutch Plant Protection Service. Three *Fusarium* species, *Botrytis cinerea*, *Coniothyrium* and *Phoma exigua* var. *exigua* were tested for their pathogenicity by inoculating healthy unwounded stems of *C.* 'Henryi' with spores.

Results and Discussion

All nurseries visited had wilted plants on their premises, but the frequency of wilt differed and appeared to be related to hygiene and the fungicide regime used. Most of the plants collected or sent in by post had wilted either completely or partially. Most wilted plants belonged to the early large flowering group of clematis (66%), but wilted late large flowering (12%) and small flowering (22%) were regularly encountered as well. *P. clematidina* was recovered from plant material from all British nurseries except one. Overall, the fungus was isolated from 23% of the plants investigated, which appears relatively low but is probably due to the slow growing character of the fungus *in vitro*. Most of these plants had wilted (89%), but *P. clematidina* was present in one plant with just leaf spots and two plants without fresh growth with severe stembase and root rot as well. The fungus was most commonly isolated from stems and less frequently found in roots. Root infection of clematis by *P. clematidina* had not been described before. Occurrence of the fungus in leaves and petioles was sporadic. The percentage recovery of *P. clematidina* for plants from private gardens (44%) was much higher than that for nurseries (18%). Apart from *P. clematidina*, nine other *Phoma* species were found, some of which have been reported in the literature as opportunistic pathogens of clematis such as *P. exigua* and *P. pomorum*. Other fungi found included *Coniothyrium*, which has been reported in the Netherlands as a cause of clematis wilt (Blok, 1965), three species of *Fusarium*, and *Phytophthora*, which was found especially on roots of small flowered species. However, none of the fungi tested for pathogenicity caused wilting symptoms in healthy clematis and most were probably secondary. Apart from *Botrytis cinerea*, which was widespread, *P. clematidina* was the most commonly found species in wilted clematis. Together with findings in earlier research (Ebben & Last, 1966; Wolff, 1996), this confirmed *P. clematidina* as the most common cause of clematis wilt in the UK. Pathogenicity tests with *P. clematidina* are described in Section 6.

©1999 Horticultural Development Council

4. GROWTH OF *PHOMA CLEMATIDINA* ON AGAR

Aim

To determine the effect of culture medium, isolate, temperature and light on growth of *P. clematidina in vitro* and compare the fungal morphology observed with that described in the literature.

Methods

Petri dishes with eight different agar media were inoculated with mycelial plugs of an isolate of *P. clematidina*. The media used were: clematis leaf agar (CLA), glucose agar (GA), malt agar (MA), oatmeal agar (OA), potato carrot agar (PCR), potato dextrose agar (PDA), V8 agar (V8A) and water agar (WA). In a second trial, mycelial plugs of ten different British isolates of *P. clematidina* were placed on V8A plates. In both experiments, the agar plates were incubated at 20°C in the dark and the diameter of mycelial growth was measured after seven days and fungal morphology studies using light microscopy.

Mycelial plugs of one isolate of *P. clematidina* were placed on V8A plates. Some were incubated in the dark at four different temperatures 5, 15, 20 and 25°C, others were placed in a glasshouse either in the dark or under natural daylight supplemented by lamps providing a 12 h photoperiod. In both cases, the diameter of mycelial growth was measured after two weeks. Results were statistically analysed using ANOVA for overall effects and Duncan's Multiple Range test or Mann-Whitney U-tests for individual differences.

Results

Although *P. clematidina* grew well on all growth media tested, the medium used did have a significant effect on the mycelial growth rate measured over one week ($\alpha = 0.05$). Mycelial growth on malt agar (MA), potato dextrose agar (PDA) and water agar (WA) was significantly slower than on the other media ($\alpha = 0.05$), while it was fastest on clematis leaf agar (CLA) and oatmeal agar (OA). However, on CLA the mycelium was thin and few pycnidia were formed, and a similar appearance was found on WA. Sporulation was abundant on MA, PDA and V8A, where the pycnidial density was high. Conidia were usually exuded in a salmon coloured, sometimes cream or orange coloured mass. The aerial mycelium was generally white, but more greyish green coloured on oatmeal agar (OA) and potato carrot agar (PCA). Especially on PCA, many chlamydospores were formed within two weeks after inoculation.

Clear differences in mycelial growth rate, sporulation and colony morphology could be distinguished between the different isolates of *P. clematidina* tested. The mycelial growth rate after one week depended significantly on the isolate used ($\alpha = 0.05$).

©1999 Horticultural Development Council

Three types of colony morphology could be distinguished:

Group I. This group showed on average a significantly slower growth rate than the other two groups ($\alpha = 0.05$), forming few pycnidia and often dense mycelium, both aerial and submerged. Pycnidia turned dark rapidly and large numbers of chlamydospores were formed.

Group II. This group formed even fewer pycnidia than group I and produced mainly dense white mycelium with few chlamydospores.

Group III. This group was distinguished by a high density of pycnidia and abundant salmon coloured spore mass with little loose aerial mycelium.

All benzimidazole resistant isolates (see Section 7) of *P. clematidina* fell in groups I and II, while all benzimidazole sensitive isolates belonged to group III.

The fungal structures formed *in vitro* were similar for all isolates. Recently formed mycelium was hyaline, but dark, thicker strands soon appeared in which eventually chlamydospores developed. Typically, chlamydospore morphology was similar to the description by Boerema (1993). There were no clear differences in chlamydospore morphology between isolates. In some isolates, strands of chlamydospores eventually formed dark structures resembling pseudosclerotia (± 250 x 75 µm). Pycnidia appeared either singly or in small, fused groups and had one ostiole each, normally without setae (hairs). Conidial exudate was copious, mostly salmon coloured. Pycnidia were light brown, but turned darker with age. On very old plates (15 months) non-typical complex pycnidial structures were formed. Conidia were mostly one-, occasionally two-celled, but in some isolates even three- or four-celled spores occurred (up to 18 x 2 µm). Apart from these multi-celled spores, the shape and size of pycnidia and spores were as reported by Boerema (1993).

Micropycnidia (diameter max. 50 µm) were found to occur in the aerial mycelium. Regularly, structures were observed in the mycelium, which according to the description given by Monte *et al.* (1989) for *P. betae*, could be pycnidial primordia. Under moist conditions, some mycelial strands ended in water filled, thin-walled vesicles (diameter 20 µm). Most isolates formed needle-shaped crystals (up to 3 cm in length) in the agar, which became especially apparent after incubation for more than three months when plates began to dry out.

Temperature had a significant effect on the growth rate *in vitro* ($\alpha = 0.05$). All plates showed fungal growth after 3 days, except at 5°C where growth was very slow. At this temperature the mycelium was white, thin and curly, while at 25°C it was grey and coarse. Almost no aerial mycelium was formed at 20°C. Numerous pycnidia exuding a salmon coloured spore mass were observed after 6 days at 15, 20 and 25°C, but no reproductive structures had formed at 5°C after 14 days. The oldest pycnidia formed at the three highest temperatures turned black and this happened especially quickly at 25°C. The average mycelial growth rate of *P. clematidina* plotted against temperature resulted in an S-curve with an optimum around 21°C.

©1999 Horticultural Development Council

After prolonged incubation (6 months) at 5°C, colonies had fully grown at this temperature as well and had formed some pycnidia with spore mass.

The average growth rate in the light was significantly higher than in the dark ($\alpha = 0.05$). Calculated over 15 days, the average growth rate was 0.30 cm/day in total darkness and 0.38 cm/day under the influence of light. In both treatments, abundant salmon coloured spore mass was exuded by the pycnidia. The plates in the dark produced somewhat more spore mass than the ones in the light, but in both treatments the pycnidia were mainly formed in distinctive bands separated by areas with mostly mycelium.

Discussion

Growth media supporting fast mycelial growth did not necessarily stimulate the formation of pycnidia. MA increased the production of pycnidia and spore mass, while OA promoted mycelial growth, which is in accordance with work on *P. clematidina* by Ebben & Last (1966). Even on the media promoting the fastest growth and under optimal growing conditions, *P. clematidina* grew slowly compared to some other *Phoma* species and some commonly used media such as MA and PDA reduced the mycelial growth rate even more. This may explain why Bemann (1968) and Wolff (1996), who used MA and PDA for isolations, had trouble in recovering *P. clematidina* from plant material, since these media reduce the chance of the fungus outgrowing competitive species even more. The salmon coloured spore mass produced by *P. clematidina* on most media aids early recognition of the species. For *P. clematidina*, the optimum balance between mycelial growth and sporulation was found on V8A, where mycelial growth was fast but took place without the formation of large amounts of aerial mycelium. This meant that the large amounts of spore mass formed could be harvested without the need to filter out mycelial strands, which facilitated the preparation of spore solutions. Since V8A is also relatively cheap and simple to prepare, it was chosen as the medium for isolation and for the provision of inoculum of *P. clematidina* in all further experiments.

Mycelial growth rate is often used as a tool for identifying *Phoma* species, but in some cases the range of values can be quite large. The values found for the isolate used in the media experiment fell within the ranges for *P. clematidina* reported by Boerema (1993). The isolates tested on V8A fell into three distinct groups with regard to colony morphology and this was found to relate to benzimidazole resistance *in vitro*. It appears that the development of benzimidazole resistance in a fungal isolate influences its ability to reproduce under normal cultural conditions, but an inhibiting effect on mycelial growth rate was found only in group I. Chlamydospore morphology in all isolates investigated was as described by Boerema (1993) and this appears therefore to be a useful, stable characteristic for identification. The crystals formed *in vitro* may be the toxin produced by *P. clematidina* (Smith *et al.*, 1994). Smith *et al.* (1994) have suggested this toxin might be ascochitine, which is also produced by a range of *Ascochyta* species.

The optimum temperature, 20-21°C, for mycelial growth of *P. clematidina* agrees with optimum temperatures for temperate *Phoma* species, although more thermophilic species exist with optima of 25°C or higher.

©1999 Horticultural Development Council

Pycnidia were formed at all temperatures, even at 5°C after prolonged incubation, which is not always the case in *Phoma* species. *P. clematidina* appeared to be well adapted to low temperatures, which might be an important factor in fungal survival in winter.

Several authors have implied that concentric growth of pycnidia in *Phoma in vitro* is induced by alternating light and dark (e.g. Wolff, 1996). However, the results in the present study suggest that changes in temperature can induce concentric distribution of pycnidia as well, since plates in constant darkness also showed this characteristic. This corresponds with results of Hafiz (1951), who found for *Ascochyta rabiei* (Pass.) Labrousse, that zonation was mainly induced by fluctuations in temperature. *P. clematidina* produced in the dark somewhat more spore mass than in alternating light/dark. However, it might also have been an effect of differences in humidity as a result of the aluminium foil wrapping. Like most *Phoma* species, *P. clematidina* clearly does not need light to produce pycnidia and spore mass, contrary to claims by Wolff (1996), who found no sporulation in this fungus when cultured in the dark.

©1999 Horticultural Development Council

5. SPORE GERMINATION AND INFECTION STUDIES WITH *PHOMA CLEMATIDINA*

5.1 Effect of different factors on spore germination in water

Aim

To study the influence of temperature, spore concentration and nutrients on the process of germination of conidia in *P. clematidina*.

Methods

Spore suspensions in water (10^5 spores/ml) were made using a benzimidazole sensitive and a benzimidazole resistant isolate of *P. clematidina*. Drops (20 μl) of the spore suspensions were placed on clean glass slides, covered with cover slips and incubated in sealed Petri-dishes containing moist filter paper at five different temperatures (5, 15, 20, 25 and 35°C). Two replicate experiments were performed for each temperature and observations were made at t = 24, 48 and 72 h.

In a second experiment, spore suspensions in water of an isolate of *P. clematidina* were prepared at four different concentrations, 10^4, 10^5, 10^6 and 10^7 spores/ml, and incubated at 20°C. Three replicate experiments were performed for each spore concentration and observations were made at t = 24 and 48 h.

In a third experiment, drops of a spore suspension (2 x 10^5 spores/ml) of an isolate of *P. clematidina* were incubated in the same way at 20°C after mixing them (1:1 v/v) with one of the following:

1. filtered V8-juice suspension (3%)
2. sucrose solution (3%)
3. sap from squashed leaves of *C.* 'Henryi'
4. sap from squashed leaves of *C. montana* 'Grandiflora'
5. leachates from a leaf of *C.* 'Henryi'
6. leachates from a leaf of *C. montana* 'Grandiflora'

The control was diluted with distilled water. Clematis sap was obtained by grinding 5.0 g of fresh leaf material in 100 ml distilled water and filtering the suspension through filter paper. The leachates were obtained by submerging a leaf attached to the plant in 25 ml of distilled water in a plastic bag for 15 h. Four replicate experiments were performed for each treatment and observations were made at t = 24 and 48 h.

In all three experiments, only spores with germ tubes ≥ 5 μm were considered to have germinated and the number was expressed as a percentage of the total number of spores counted per treatment (n = 50). Septation and number of germ tubes formed by each spore were noted in the first two experiments. Results were statistically analysed using ANOVA for overall effects and Duncan's Multiple Range test or Mann-Whitney U-tests for individual differences. In case of frequencies, χ^2- tests were used.

©1999 Horticultural Development Council

Results

The overall effect of temperature on spore germination was significant at all three time intervals for both isolates of *P. clematidina* ($\alpha = 0.05$). A significant difference was detected between the benzimidazole sensitive and resistant isolate at all three time intervals ($\alpha = 0.05$), since spore germination was quicker at lower temperatures for the resistant isolate. For both isolates, the average percentage germination was optimal at 20°C reaching almost 100% after 48 h. At other temperatures this optimum had not been reached after 72 h. Germination percentages at 15°C and 25°C were very similar, especially for the benzimidazole resistant isolate, and reached at least 80% after 72 h. Almost no germination was observed at 35°C at which temperature the spores quickly disintegrated. At 5°C, the germination rate was low and reached a maximum of 52% after 72 h, but after prolonged incubation (10 days) 75 and 88% of conidia respectively germinated.

Spore concentration had a significant effect on the percentage spore germination both at 24 and 48 h ($\alpha = 0.05$). Over the first 24 h, germination was fastest at a concentration of 10^5 spores per ml, but 24 h later the highest percentage was found at a concentration of 10^4 spores per ml. With 10^6 spores per ml, some spores (11%) germinated in the first 24 h, but no further germination took place after that. At the highest spore concentration, almost no germination occurred and the spores had not even started to swell at 48 h. The variation in percentage germination between the different replicates was for the lowest three concentrations in most cases rather large. This meant that no significant differences could be detected between the concentrations 10^4 and 10^5 spores per ml and between 10^6 and 10^7 spores per ml at either time interval ($\alpha = 0.05$). However, the two lowest concentrations did differ significantly from 10^6 spores per ml at 48 h, and 10^7 spores per ml at both time intervals ($\alpha = 0.05$).

There was a significant effect of nutrient solution on the percentage germination, both at 24 and 48 h ($\alpha = 0.05$). The percentage germination in the control with water was relatively low, only 9% after 24 h. This meant that the influence of nutrients on spore germination could be easily assessed. The filtered V8-juice had the most stimulating effect on spore germination and after 24 h, 89% of the spores in this solution had already germinated and were strongly swollen. Spore germination in both leachate and sap of *C. montana* was significantly higher than in the same solutions for *C.* 'Henryi' at both time intervals ($\alpha = 0.05$). For both varieties, the sap had a significantly more stimulating effect than the leachate ($\alpha = 0.05$). After 48 h, no significant difference between leachate from the susceptible variety and water could be detected, while the same was true for leachate of the resistant variety and the sucrose solution. Significant differences in percentage germination between sap from *C.* 'Henryi' and sucrose or sap of *C. montana* and V8-juice could not be detected at this time interval either ($\alpha = 0.05$).

There was a significant difference in percentage spores with multiple germ tubes between the different nutrient solutions, but only at 48 h. ($\alpha = 0.05$). The effect on the average percentage of germinated spores with more than one germ tube was almost the opposite to that for spore germination.

©1999 Horticultural Development Council

After 48 h, filtered V8-juice had resulted in significantly fewer germ tubes per germinated spore than all of the other solutions ($\alpha = 0.05$), while the highest average was found in water. Sap from *C.* 'Henryi' significantly increased the number of germ tubes per spore in comparison with sap from *C. montana* 'Grandiflora', but no significant difference was detected between the leachates ($\alpha = 0.05$). No significant difference in effect on germ tube number between sap and leachate of *C.* 'Henryi' was found, but the leachate of *C. montana* had a significantly greater effect than its sap ($\alpha = 0.05$).

Germ tube length in sap from both varieties reached up to 350 μm at 48 h, as did those in leachate from *C. montana* and in sucrose. However, germ tubes in leachate from *C.* 'Henryi' remained very short and did not reach more than 35 μm at 48 h, which was similar to those in water. Germ tubes in V8-juice were generally much longer than with the other solutions and reached up to 1.7 mm at 48 h. For all treatments, the average percentage two-celled spores was higher at 48 h than at 24 h indicating that septation was in many cases secondary. The difference in germination between one- and two-celled spores usually also included a difference in degree of swelling. Nutrient solution had a significant effect on the number of two-celled spores at 48 h ($\alpha = 0.05$). The average percentage of two-celled spores at 48 h was significantly higher in water (11%) and sap (12%) and leachate (13%) from *C.* 'Henryi', than in V8-juice (2%), sucrose (7%) and sap (2%) and leachate (7%) from *C. montana* 'Grandiflora' ($\alpha = 0.05$).

Discussion

The results of the *in vitro* experiments showed that spore germination in *P. clematidina* is influenced by temperature, spore concentration and the availability of nutrients. Germination takes place in water drops on glass slides in the absence of exogenous nutrients and at a wide range of temperatures. Increasing spore concentrations reduce the percentage spore germination in many fungal species (Macko *et al.*, 1976), and the inhibition of germination at higher concentrations, as observed for *P. clematidina* in these studies, agrees with results obtained by Smith (1987). At the highest concentration tested, 10^7 spores per ml, very few spores germinated. There are indications that *Phoma* spores excrete a chemical preventing germination, but the inhibition of germination at high spore concentrations has also been attributed to inhibitory effects of the matrix in which the spores are exuded by the pycnidia. However, Smith (1987) found that removal of the matrix by washing spores of *P. clematidina* reduced rather than increased the percentage germination. The optimum concentration for spore germination *in vitro* for *P. clematidina*, 10^4 to 10^5 spore per ml, corresponds with the one found by Smith (1987). However, the optimum spore concentration for germination *in vitro* and that for infection *in vivo* are not necessarily the same.

Although germination of *Phoma* spores may take place in water without exogenous nutrients, the results of the experiments showed that the percentage of germinating spores can be very variable under these conditions. This is why some researchers add fruit juice to spore suspensions in order to obtain consistently high germination rates (e.g. Vanniasingham & Gilligan, 1988). The results of the nutrient experiment showed that water with V8-juice increased the number of germinated spores significantly compared to plain water or water with sucrose, and resulted in a rapid growth of germ tubes.

©1999 Horticultural Development Council

In conidia of *P. clematidina*, both secondary septation and the formation of multiple germ tubes were found to be induced by a lack of exogenous nutrients. The percentage of spores forming a septum was generally much lower (max. 13% after 48 h) than the percentage of spores forming multiple germ tubes (max. 90% after 48 h), but in both cases the percentage was significantly higher in plain water than in the presence of V8-juice or sucrose. The results suggest that leaves of *C.* 'Henryi' contain less nutrients than those of *C. montana* 'Grandiflora', which may influence both spore germination and germ tube growth.

5.2 Studies of spore germination on leaves at light microscopy level

Aim

To study the sequence of events in germination of conidia of *P. clematidina* on the surface of detached leaves of different clematis varieties.

Methods

Young, compound leaves of *C.* 'Henryi' and *C. montana* 'Tetrarose' were detached, and the petiole of each leaf was put through a hole in a plastic cap so that it dipped into tap water contained in a glass vial. The lower surface of each of seven leaves per variety were inoculated with 30 µl per leaflet of a suspension of conidia (10^5 spores/ml) of an isolate of *P. clematidina* recovered from *C.* 'Miss Bateman'. Additionally, one leaf per variety was inoculated in a similar manner on the upper leaf surface. All leaves were incubated in sealed plastic containers with moist filter paper at 20°C in the dark. At t = 2, 4, 6, 8, 10 and 12 h , one leaf of each variety was removed from the container, carefully blotted dry with filter paper, and covered on the inoculated surface with clear nail varnish. After 24 h, the remaining leaves inoculated on the lower or upper surface were also treated in this way. After the nail varnish had dried, it was peeled off and left for at least one day in a filtered solution of 0.2% cotton blue in lactic acid (60% w/w syrup). The nail varnish impressions were then washed twice in sterile distilled water to remove the residues of the stain and mounted upside down on glass slides in lactic acid. Germinating spores, stained by the cotton blue, were observed using a light microscope, and the stage of germination, presence of septation and germ tube length were assessed for two groups of 50 spores for each treatment.

Results & Discussion

The first germ tubes ≥ 5 µm were observed after 8 h, but shorter ones were present after 6 h. Extra germ tubes were not formed until after 12 h, but after 24 h most spores had formed two or three germ tubes. No clear differences in the sequence of events between 'Henryi' and *C. montana* were found, but average germ tube length at 24 h, which was 32 µm and 42 µm respectively, was significantly higher for the resistant variety. There was also a significant difference in average germ tube length at 24 h between the lower and upper surface, which was 29 µm and 44 µm respectively. The stage of germination and average length of germ tubes differed considerably between groups of spores, even for the same treatments. Germ tubes rarely grew directly towards stomata.

©1999 Horticultural Development Council

Due to the small size of *P. clematidina* spores, special infection structures such as appressoria or other infection structures could not be distinguished using light microscopy. Thus more detailed studies were made using Scanning Electron Microscopy (SEM).

5.3 Studies of spore germination and infection at SEM level

Aim

To study the host-pathogen interaction between two clematis varieties differing in wilt resistance and *P. clematidina,* and to identify possible resistance mechanisms.

Methods

Different preparation techniques for Scanning Electron Microscopy (SEM) were tried on young, uninfected leaves of *C.* 'Henryi' and *C. montana* 'Grandiflora' in order to establish the best method for the preservation of surface features. The following techniques were compared:

(a). Air drying (AD). Pieces of untreated leaf were air dried for 12 h at 25°C in a desiccator.

(b). Critical point drying (CPD) without washing. Pieces of untreated leaf were fixed in 4% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 hours at 4°C, then washed three times (10 minutes each) in phosphate buffer, and fixed in 1% osmium tetroxide for 1 h. After another three washings in phosphate buffer, these pieces were put through a series of 30, 50, 70, 85, 95, 100 and again 100% ethanol (7 minutes each) and critical point dried in a Polaron Jumbo.

(c). Critical point drying (CPD) with washing. As under (b), but the leaf pieces were first washed for 20 sec in 70% ethanol and then sterile distilled water in order to remove any micro-organisms naturally present on the leaf surface.

All AD and CPD leaf pieces were sputter coated with gold using an EMScope SC500 before viewing in the SEM.

(d). Cryofixation/Low Temperature SEM (LTSEM). Pieces of untreated leaf were mounted on an aluminium specimen holder attached to a brass 'shuttle' using a mix of colloidal graphite and tissue-Tek (1:1, v/v), and plunged into liquid nitrogen slush. The samples were then quickly transferred to the stage of the SEM at temperatures between -190 and -170°C, and etched at - 95°C for 5 min. After etching, the samples were moved to a Polaron LT7400 cryoprep chamber at - 175°C, and sputter coated with gold/palladium. The samples were then transferred back onto the cold SEM stage for viewing.

All plant samples were viewed in a Cambridge 250/3 Scanning Electron Microscope at 15 kV. Photographs were taken with a Nikon EM camera loaded with Ilford FP4 plus film rated at ASA 125.

©1999 Horticultural Development Council

Leaves of *C.* 'Henryi' and *C. montana,* inoculated with spores of an isolate of *P. clematidina*, were prepared following methods (b) and (d) in order to compare the effect of the preparation method on the fungal structures. In addition, both non-inoculated and inoculated leaves and stems were observed in a Phillips XL30 Environmental Scanning Electron Microscope FEG. Pieces of plant material were mounted on a stub using colloidal graphite and viewed on the ESEM stage under high humidity conditions, at low vacuum, and at 10 kV.

Since LTSEM showed the best preservation of plant- and fungal structures, it was used to study further the interaction between *P. clematidina* and leaves and stems of *C.* 'Henryi' and *C. montana* 'Grandiflora'. Plants of these two varieties, which were three months old at the time of potting, were grown in a temperature controlled glasshouse (18-25°C) under natural daylight supplemented with mercury vapour lamps (max. illumination 100-500 μmol m^{-2} s^{-1}) providing a 12 h photoperiod. For inoculation, leaves and stems were sprayed with a suspension of 10^6 spores/ml of *P. clematidina*. Inoculated plants were incubated for 48 h at high humidity in sealed plastic breeding chambers in a growth room at 20°C ± 1°C on a 12 h photoperiod, with illumination provided by Phillips daylight fluorescent tubes (50 μmol m^{-2} s^{-1}). Per variety, three different plants were examined. Apart from differences in general morphology between the two varieties, the number and length of germ tubes, the presence of infection structures and other growth characteristics of the fungus on the lower leaf-, upper leaf- and stem surface were noted for 50 spores per surface per plant. Results were statistically analysed using ANOVA for overall effects and Duncan's Multiple Range test or Mann-Whitney U-tests for individual differences.

Results

All air dried samples of the clematis leaves showed greatly distorted tissues in which separate cells were often indistinguishable. This damaging effect occurred with both the upper and lower leaf surface. With the conventional method of preparation using critical point drying, most of the plant cells on the upper leaf surface appeared to retain their normal shape. However, comparison of the critical point dried lower leaf surfaces with samples which were subjected to cryofixation showed that major distortion took place during CPD as well and that with this technique the epidermal cells lost up to half of their volume due to dehydration. Comparison of leaves washed with ethanol and non-washed leaves showed that the alcohol removed most of the epidermal wax layer. Pre-fixation ethanol washings were therefore not applied to further samples, also because not many micro-organisms were found to be present naturally on the leaf surface. Regularly, post-fixation washes in ethanol during the CPD process removed large parts of the wax layer, even on the stem where thick crusts of wax were observed following cryofixation.

After CPD, fungal spores and germ tubes, as far as they were still recognisable, were usually dehydrated and deformed. Like the epidermal plant cells, the spores and germ tubes of *P. clematidina* retained more of their volume and shape when cryofixation was used. Even when spores had partly collapsed, which was observed occasionally in cryofixed samples due to dehydration during incubation or because of overetching, fungal structures were still much better preserved than with CPD.

©1999 Horticultural Development Council

Observations of the *Phoma* spores in the ESEM were also hampered by their fragile nature. The spores could not be observed at magnifications higher than ± 2000 times, since the non-fixed spores were evaporated by the electron beam within seconds at magnifications which were used without problems for fixed samples in the conventional SEM. Although the fungal structures could not be studied in detail, the ESEM observations of spore germination confirmed those made using light microscopy and LTSEM. Like the fungal spores, the unfixed plant cells also suffered very quickly from electron beam damage in the ESEM. Epidermal leaf tissue became greatly distorted compared to LTSEM samples. Due to their thickness, stem samples were less vulnerable to electron beam damage and largely retained their shape. Apart from problems with electron beam damage of delicate tissues, the ESEM delivered pictures which were much less clear than those made in the conventional SEM at the same magnification. As a result of the comparison of the different SEM techniques, it was decided to conduct further studies of the interaction between *P. clematidina* and its host using LTSEM.

In both clematis varieties examined, the lower leaf surface appeared smooth and epicuticular wax structures were observed only on the upper leaf surface and stem. The upper leaf surface of *C. montana* 'Grandiflora' bore wax crystalloids in the form of irregular platelets, while the wax on leaves of *C.* 'Henryi' occurred in irregular granules. Stems were covered with a thick crust of amorphous wax which did not greatly differ between the two varieties. Epidermal cell walls on the upper surface were strongly convex resulting in deep valleys at the cell junctions, whereas these features were less pronounced on the lower surface. This difference was especially clear for the *C. montana* leaves. Stomata, elliptical in shape, were found only on the lower leaf surface and stem. On the stems, the guard cells were not covered by the crust of wax. All plant surfaces were found to bear both long, pointed non-glandular trichomes and short, rounded, glandular trichomes. Both types were especially abundant on the surface of the stem of the susceptible variety. On leaves, the trichomes arose mostly from veins and leaf edges, and only occasionally from normal epidermal cell tissue.

Spore germination occurred on all surfaces investigated. There was a significant effect of plant surface on the percentage germination, but no significant difference was detected for the overall effect of variety ($\alpha = 0.05$). The percentage non-germinated spores encountered when scanning for germinated spores was lowest on the upper leaf- and stem surface of *C.* 'Henryi'. No significant difference in germination between the resistant and susceptible variety was found for the lower leaf surface, but the percentage germination was significantly lower for the upper leaf- and stem surface of *C. montana* than for those of *C.* 'Henryi' ($\alpha = 0.05$) (see Table 1). Germination often started with a swelling of the conidia, which regularly collapsed once germ tubes had formed. Most single celled conidia produced just one germ tube, but regularly two, three or even four germ tubes emerged from a single celled spore (see Figure 2.1). Both the percentage germinated spores with multiple germ tubes and the average germ tube length per spore were significantly higher for *C.* 'Henryi' than for *C. montana* 'Grandiflora' ($\alpha = 0.05$) (see Table 1).

©1999 Horticultural Development Council

Table 1. Percentage germinated spores, percentage germinated spores with multiple germ tubes and average total length (µm) of germ tubes of conidia of *Phoma clematidina* on the leaf- and stem surface of two clematis varieties, 48 h after inoculation, as observed with LTSEM (n = 50 germinated spores).

variety	plant surface	av. % germination for all spores[1]	% of germinated spores with multiple germ tubes[1]	av. total germ tube length per spore (µm) (± SE)[1]
C. 'Henryi'	lower leaf	55.6 a	32.0 a	3.8 ± 0.52 a
	upper leaf	96.2 b	62.0 b	59.4 ± 7.61 b
	stem	96.2 b	32.0 a	30.3 ± 4.54 c
C. montana	lower leaf	64.9 ac	14.0 c	3.1 ± 0.32 a
	upper leaf	71.4 cd	14.0 c	5.8 ± 1.72 a
	stem	84.8 d	14.0 c	11.5 ± 3.32 d

1 results in the same column not sharing a common letter are significantly different ($\alpha = 0.05$)

In addition, there was a significant overall effect of plant surface on both percentage multiple germ tubes and germ tube length. They were both significantly higher on the upper leaf surface of *C.* 'Henryi' than on any other surface. However, the differences in number and length of germ tubes between the three different plant surfaces were less pronounced for *C. montana* than for *C.* 'Henryi' (see Table 1).

The direction of emergence of germ tubes appeared to be random and not directed towards cell junctions or stomata. Penetration of the stomatal opening was never observed, even when spores landed on the guard cells. Germ tubes regularly grew over or past stomatal openings without changing shape or direction (see Figure 2.2) and never grew directly towards stomata. Conidia and germ tubes both showed signs of the presence of an extracellular matrix in places where they made contact with the plant surface.

P. clematidina was observed to enter the host tissue through direct penetration of epidermal cells as well as stomatal guard cells. A cushion shaped appressorium was the most common form of infection structure and was usually formed at the end of a germ tube. However, occasionally the appressorium emerged directly from the conidium or was formed on the side of a germ tube. Other infection structures observed were half circular or thin narrow protrusions from the germ tube tip.

©1999 Horticultural Development Council

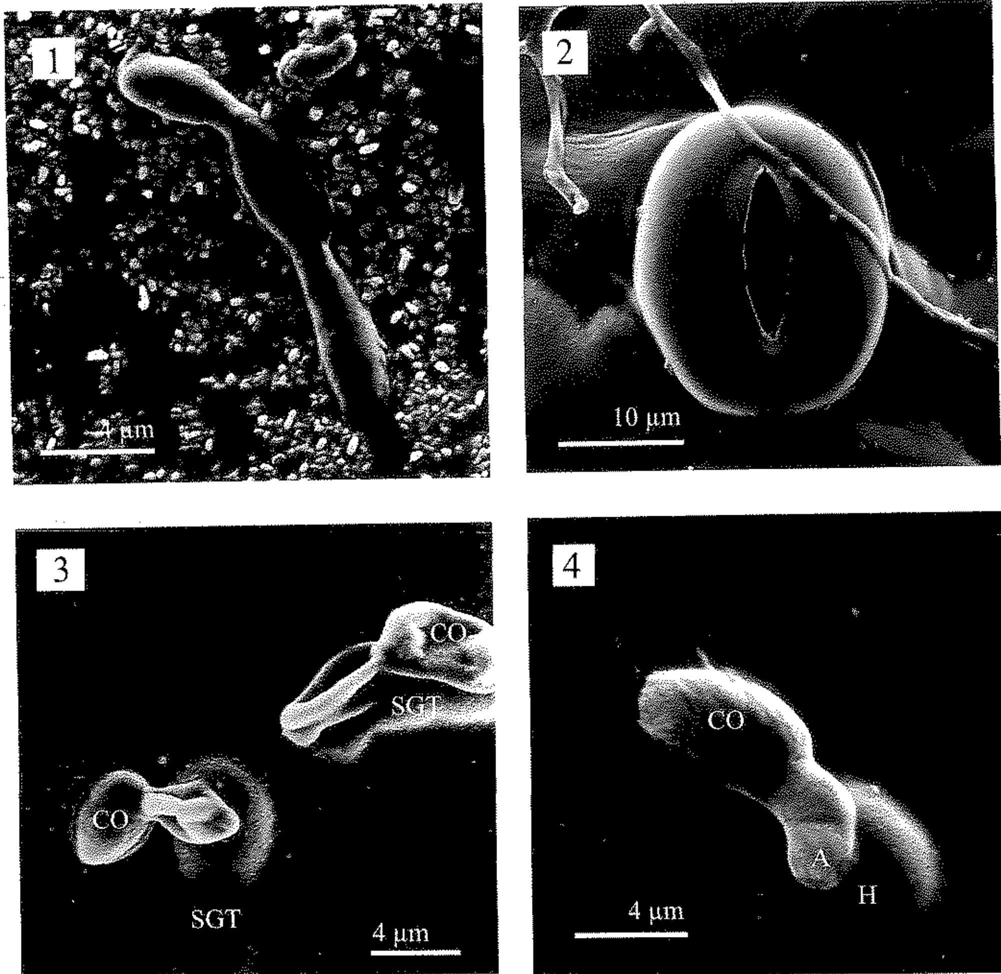


Figure 2. Scanning Electron Microscopy photographs of *Phoma clematidina* on the clematis leaf surface. 1. Spore with two germ tubes. 2. Germ tube passing near a stomatal opening. 3. Subcuticular germ tubes (SGT) on *C. montana* 'Grandiflora'. 4. Halo formation (H) at the site of appressorium (A) formation on *C. montana* 'Grandiflora'. CO = conidium.

The formation of multiple germ tubes has also been reported in *P. betae* (Monte & García-Acha, 1988) and *P. lingam* (Musa, 1981), but no indications of causal factors were given in these cases.

Germ tube length per spore was found to be higher on stem- and adaxial leaf surfaces of the susceptible variety than of the resistant species. Musa (1981) reported longer germ tubes of *P. lingam* on resistant host leaves than on susceptible leaves, but in general, resistance against fungal pathogens is connected with a lack of growth on the plant surface.

Comparison of observations of fungal development on the upper leaf- and stem surface with the lower leaf surface indicates that spore germination, the formation of multiple germ tubes, and germ tube growth were all positively influenced by the presence of epicuticular wax particles. This was found to be true for both varieties and could be explained by assuming that *P. clematidina* uses epicuticular wax structures for host recognition which then triggers spore germination and germ tube growth. On the other hand, the epicuticular wax may form a mechanical barrier for the fungus, especially on the clematis stem where it formed a thick crust. Gloyer (1915) indicated that lower leaf surfaces of clematis were more easily infected than upper surfaces. Since *P. clematidina* obviously does not enter its host through stomata, the difference in amount of epicuticular wax may explain this observation.

The general behaviour of *P. clematidina* on the surface of clematis plants showed several aspects, such as attachment by the formation of an extracellular matrix and germ tube tropism in reaction to cell junctions, which are also found in many other fungal species (Wynn, 1981). The pre-penetration events in *P. clematidina* are in many respects similar to those in *P. lingam* described by Musa (1981). In both species, a single conidial cell can form multiple germ tubes, germ tube orientation is random, and penetration may take place without the formation of infection structures. Although direct penetration does occur in *P. lingam*, it usually enters its host through stomata (Musa, 1981), which was never observed for *P. clematidina* in the present study. In that respect, the behaviour of *P. clematidina* is more in accordance with that of *P. tracheiphila* on lemon leaves (Zucker & Catara, 1986). In contrast to *P. tracheiphila*, *P. clematidina* does not need wounds to enter its host, despite suggestions by Smith & Cole (1991) to the contrary. Apart from differences in percentage spore germination, number of germ tubes per spore and germ tube length between the susceptible and resistant varieties, there was also a difference in the percentage of germ tubes which formed infection structures on the plant surface. The fact that infection structures were more common on the resistant variety agrees again with data for *P. lingam* (Musa, 1981). Like spore germination, germ tube formation and growth, the formation of infection structures may be promoted by the availability of nutrients, which may explain differences between the varieties. However, the fungus may also have been forced to form more infection structures because of a higher number of failed penetration attempts due to resistance of the host tissues.

Subcuticular growth of germ tubes occurs in a range of plant pathogens including *P. lingam* (Musa, 1981) and several species of *Ascochyta* (e.g. Clulow *et al.*, 1991). In contrast to the observations made in *P. clematidina*, subcuticular growth in these fungi occurs on both susceptible and resistant host plants.

©1999 Horticultural Development Council

Subcuticular growth in *P. clematidina* could be the result of a resistance reaction in the epidermal cell layer of the host, such as a partial blockage of intercellular spaces by lignification. This idea supported by the formation of haloes in the plant cuticle of the resistant clematis variety at the site of infection structure formation by *P. clematidina*, since in other pathosystems, halo formation has been associated with papilla deposition (e.g. Bird & Ride, 1981).

The differences in events on the surface of plants differing in susceptibility suggest, that the defence of small flowered clematis against *P. clematidina* starts even before the fungus attempts penetration. This means that resistance of host cells towards the fungal toxin ascochitine and the abscission of infected leaves (Smith & Cole, 1991; Smith *et al.*, 1994), are not the only resistance mechanisms of small flowered clematis against *P. clematidina*, and that they become important only if the fungus first succeeds in germination, germ tube growth, infection structure formation and penetration of the epidermis.

Many of the observations discussed above were made using LTSEM. This method was chosen because both plant- and fungal structures retained more of their original volume and shape than with CPD. Although the ESEM saved time during sample preparation and was usable for the observation of some plant material such as stems, the present studies showed that LTSEM generally gives much better results. The quality of the observations and photographs achieved using LTSEM compared to ESEM made up for the time spent on fixation and coating of the samples.

©1999 Horticultural Development Council

6. DISEASE DEVELOPMENT STUDIES WITH *PHOMA CLEMATIDINA*

6.1 Virulence of different fungal isolates

Aim

To establish whether there are differences in pathogenicity or virulence between isolates of *P. clematidina*.

Methods

Five benzimidazole resistant and five benzimidazole sensitive isolates of *P. clematidina* were tested on leaves of six months old plants of *C.* 'Henryi'. One young, completely expanded leaf or leaflet was inoculated with a mycelial plug of one of the ten isolates recovered from wilted clematis plants in the UK, or as a control with a sterile agar plug, and then wrapped in a plastic bag to maintain high humidity. Leaf spot diameter (mm) was regularly measured over 90 days. Results were statistically analysed using ANOVA.

Results & Discussion

A clear difference in virulence between the group of benzimidazole sensitive isolates and the group of benzimidazole resistant isolates of *P. clematidina* was detected. Both disease incidence and average leaf spot diameter increased much more slowly for the benzimidazole resistant group. After 90 days, both the percentage of plants with leaf spots larger than 1 mm and the average leaf spot diameter were significantly higher for the group of benzimidazole sensitive isolates ($\alpha = 0.05$). In case of the benzimidazole resistant isolates, leaf spots were often formed at the leaf edge where the plant exudes nutrients which may promote spore germination and fungal growth. However, after 90 days all isolates had caused leaf spot symptoms, which meant that all isolates tested were pathogenic. These studies showed that virulence is negatively correlated with benzimidazole resistance. This could be related to a lack of enzymes such as cutinase needed to penetrate the plant cuticle, phytoalexin detoxifying enzymes, or fungal toxins needed to kill the host cells, such as ascochitine (Clarkson, 1992; Smith *et al.*, 1994).

6.2 Disease development on leaves and stems of two varieties

Aim

To determine the effect of different inoculation methods on susceptibility of leaves and stems of two clematis varieties differing in wilt resistance to infection by *P. clematidina*, and to determine whether the reaction of detached leaves and stems could be correlated with the level of infection on when leaves and stems remained attached to the plant.

©1999 Horticultural Development Council

Methods

Unwounded, young, fully extended leaves and non-woody internodal stems of six months old plants of *C.* 'Henryi' and *C. montana* 'Grandiflora' were inoculated with either a mycelial plug of *P. clematidina* or with filter paper with spore suspension, and wrapped in plastic to maintain high humidity. Controls were inoculated with sterile agar plugs or filter paper with sterile distilled water. Only one leaf or stem per plant was used. At the same time, from each plant inoculated with spores or the corresponding control, one uninfected leaf or stem was cut off, inoculated in the same way as the attached leaf or stem, and incubated in a plastic box. Leaf spots were measured regularly to determine their greatest width and stems checked for wilting symptoms. After the trial, leaf and stem pieces showing symptoms were plated out on V8A to check for the presence of *P. clematidina*. Results were statistically analysed using ANOVA for overall effects and Duncan's Multiple Range test or Mann-Whitney U-tests for individual differences. In case of frequencies, χ^2- tests were used.

Results

Leaf spots were formed both on *C.* 'Henryi' and *C. montana* 'Grandiflora', but on the resistant variety they usually remained small in size ($\leq$ 2 mm) and only occasionally grew into larger spots. On *C.* 'Henryi' the spots developed much more rapidly and in most cases the fungus eventually invaded the whole leaf or leaflet. Two heavily infected leaves of *C. montana* abcissed, but most remained on the plant. Both clematis variety and inoculation method had a significant effect on the average leaf spot diameter by day 70. However, the percentage of diseased leaves was affected significantly only by inoculation method and no significant differences could be detected between individual treatments ($\alpha = 0.05$). Inoculation with 10^6 spores per leaf was the most effective method in both clematis varieties, but particularly in *C.* 'Henryi' where the average leaf spot diameter at day 70 were significantly higher with this inoculation method than with agar plugs ($\alpha = 0.05$). Disease development was quicker and symptoms were more severe in the detached leaves compared to the attached leaves with the same inoculum, but leaf spot appearance was similar. Detached leaves of *C. montana* were generally stronger and stayed fresh for longer than those of *C.* 'Henryi', some of which began to yellow within the duration of the experiment.

After 30 days, all attached *C.* 'Henryi' stems except one had wilted from the point of inoculation upwards. Those inoculated with mycelium wilted significantly quicker (first one after 11 days) than those inoculated with spores (first one after 15 days) ($\alpha = 0.05$), but the percentage of wilted plants was slightly higher with the spore inoculum. Of the 20 *C. montana* stems inoculated, only two stems inoculated with mycelium had wilted after 30 days. All other inoculated *C. montana*'s as well as all the controls did not show any signs of wilt. There was a significant effect of clematis variety on percentage wilted plants, but no significant difference was detected for the average time interval before wilting. The significance of the effect of inoculation method was just the reverse ($\alpha = 0.05$). Wilted stems which were cut open showed a distinctive, black discolouration of the vascular tissues. In *C.* 'Henryi, the infection had usually spread over the whole length of the stem covered by the moist paper (± 8 cm) and sometimes even beyond, up- and downwards from the point of inoculation.

©1999 Horticultural Development Council

In *C. montana*, the lesions were much more limited. The bark was often starting to separate from the inner tissues and white mycelium could be seen in the space thus created. Pycnidia were observed on the bark of most of the detached *C.* 'Henryi' stems inoculated, but black discolouration of the vascular tissues was not found.

Discussion

The results of these experiments confirmed *P. clematidina* as a cause of leaf spot, stem rot and wilt in clematis. The fungus easily infected unwounded tissues. Both leaves and stems of *C.* 'Henryi' were highly susceptible to infection by *P. clematidina*, which corresponds with its susceptibility to wilt in practice (see Chapter 2). Leaf spots expanded rapidly in contrast to those on *C. montana*, where lesions remained limited in most cases. Only occasionally, *P. clematidina* seemed to succeed in breaking the resistance barrier of *C. montana* leaflets to invade the whole leaf. The fact that this happened more often in detached leaves than attached ones indicated that the physiology of the leaves had changed after detachment and that the detached leaf test did not reflect the situation in whole plants. Whether the small black lesions observed on *C. montana* represented sites where *P. clematidina* actually penetrated the plant tissues could not be determined. The lesions could have been due to localised cell death as part of a hypersensitive reaction before fungal penetration took place. Similar lesions have been described for resistant host plants in reaction to conidia of several *Ascochyta* species (e.g. Darby *et al.*, 1986). Abscission of infected leaves of *C. montana*, reported as a resistance mechanism in this clematis species by Smith & Cole (1991), was observed only twice and thus appears to be important only if *P. clematidina* breaks through other resistance barriers.

As with leaf inoculation, the stem inoculation experiment showed a clear difference in susceptibility between *C.* 'Henryi' and *C. montana*. Unlike leaf infection, successful stem infection had a highly destructive effect on plant health and resulted quickly in the symptoms which are typical of clematis wilt. It is not surprising that to many growers clematis wilt appears to be a sudden event since early stem infection is not easily recognised. Infected stems did not change shape and appeared almost normal on the outside. Although the difference in susceptibility between *C.* 'Henryi' and *C. montana* also became apparent in the detached stems, the symptoms were not as extensive as in the attached stems. Wilt symptoms could not be observed in detached stems which are therefore not very suitable for testing host susceptibility to *P. clematidina*.

6.3 Disease development on roots

Aim

To determine the susceptibility of clematis roots to infection by *P. clematidina*.

Methods

C. 'Henryi' plants were removed from their containers and their roots washed clean. The plants were then placed in 250 ml glass flasks filled with 200 ml sterilised distilled water such that only the lower part of the root system was submerged.

©1999 Horticultural Development Council

Ten flasks were inoculated with V8A plugs containing pycnidia and spore mass of *P. clematidina*. Ten control plants were inoculated with sterile agar plugs. The water level was kept constant by adding sterilised distilled water every two to three days. The plants were checked daily for disease symptoms and roots of wilted plants were plated out on V8A agar to check for the presence of *P. clematidina*. After 100 days the experiment was terminated and the roots of non-wilted plants were plated out as well.

Results & Discussion

About three weeks after inoculation of the healthy root systems with *P. clematidina*, the first signs of root infection became apparent. Small black lesions started to form on the roots of all inoculated plants, especially at the interface of water and air. The lesions expanded and eventually whole roots rotted away with the disease spreading slowly through the root system. In some cases pycnidia with pink coloured spore mass could be detected on the black lesions.

Plants in which the remaining roots could no longer provide enough water wilted. Seven of the ten inoculated plants died within the duration of the experiment (100 days), taking on average 52 days to wilt. The first plants wilted after 35 days. *P. clematidina* was isolated from all inoculated plants, also from the three plants that had not wilted after 100 days. Because of the unfavourable growing conditions (hot, dry weather, no shade), four of the control plants wilted as well, the first one after 45 days and on average after 63 days. However, none of them showed any sign of black root lesions or yielded *P. clematidina* when plated out.

Koch's postulates were established for root rot of clematis caused by *P. clematidina*. As indicated in Chapter 3, root rot in clematis caused by *P. clematidina* has not been reported before. Gloyer (1915) observed only limited lesions when he inoculated clematis roots with *P. clematidina*, but in the present studies the fungus was found to be capable of causing severe root rot. Most workers on *P. clematidina* have not investigated this aspect of clematis wilt, even though many other *Phoma* species are known as root pathogens. The results confirm the observations described in Section 3 that *P. clematidina* can be present on rotted roots of wilted containerised clematis plants on nurseries. The effect of growing medium on root infection of clematis by *P. clematidina* is described in Section 7.5.

6.4 Disease development in a range of varieties

Aim

To investigate whether the reaction of clematis varieties to inoculation of stems with *P. clematidina* forms a good indication of their susceptibility to wilt in practice.

Methods

Non-woody stems of clematis plants of 11 different varieties (see Table 3) were inoculated with a mix of spores of six different isolates of *P. clematidina* by pipetting 0.2 ml of a spore suspension of 5 x 10^6 spores per ml on a piece of filter paper.

©1999 Horticultural Development Council

The filter paper was placed against an unwounded internodal section of a stem, wrapped in moist tissue paper and sealed with laboratory film. Controls were inoculated with filter paper with sterile distilled water. Only one stem per plant was used and there were ten replicates per treatment. Plants were checked daily for wilting symptoms for 60 days. The plants were kept in two litre containers in a ventilated polytunnel, shaded by green netting and placed in randomised blocks.

Table 3. Clematis varieties tested on susceptibility to *Phoma clematidina* by inoculation of unwounded stems with a mix of conidia of six different fungal isolates.

group	variety	parentage[1]
early large flowering	*C.* 'Henryi'	*C. lanuginosa* x *C. fortunei*
	C. 'Miss Bateman'	*C. standishi* x *C. fortunei*
	C. 'Vyvyan Pennell'	*C.* 'Daniel Deronda' (*C. lanuginosa* x *C. patens*) x *C.* 'Beauty of Worcester' (*C. lanuginosa* type)
late large flowering	*C.* 'Comtesse de Bouchaud'	*C.* 'Star of India' (*C. lanuginosa* x *C.* 'Jackmanii' [*C. viticella* x *C. lanuginosa*]) x *C. texensis*
	C. 'Ernest Markham'	*C. viticella* type
	C. 'Lady Betty Balfour'	*C.* 'Gypsy Queen' (*C. patens* x *C.* 'Jackmanii' [*C. viticella* x *C. lanuginosa*]) x *C.* 'Beauty of Worcester' (*C. lanuginosa* type)
	C. 'Ville de Lyon'	*C. viticella* type x *C. texensis*
early small flowering	*C. alpina* 'Frankie'	
	C. montana 'Grandiflora'	
late small flowering	*C. orientalis* 'Bill Mackenzie'	
	C. viticella 'Purpurea Plena Elegans'	

1 after Howells (1994a)

Results were statistically analysed using ANOVA for overall effects and Duncan's Multiple Range test or Mann-Whitney U-tests for individual differences. In case of frequencies, χ^2- tests were used.

©1999 Horticultural Development Council

Results

There was a significant effect of clematis variety on both percentage of wilted plants after 60 days and average time before wilting ($\alpha = 0.05$). The first signs of wilt were observed after 12 days in *C.* 'Miss Bateman' and *C.* 'Lady Betty Balfour'. Many more plants of different varieties followed, and after 60 days, 49% of all inoculated plants had wilted (see Table 4).

Table 4. Effect of clematis variety and flowering group on percentage wilted plants and average time interval before wilting after inoculation of unwounded internodal stems with conidia of *Phoma clematidina*, 60 days after inoculation (n = 10).

group/ variety	average time before wilting (days) ± S.E.[1]		% wilted plants[1]	
Early large flowering group				
C. 'Henryi'	36.9 ± 3.5	a	70	abc
C. 'Miss Bateman'	33.6 ± 8.3	a	50	ac
C. 'Vyvyan Pennell'	36.4 ± 3.4	a	90	ab
group average	35.9 ± 2.6		70	
Late large flowering group				
C. 'Comtesse de Bouchaud'	41.6 ± 1.6	b	50	ac
C. 'Ernest Markham'	26.9 ± 3.9	c	100	b
C. 'Lady Betty Balfour'	26.0 ± 3.0	c	70	abc
C. 'Ville de Lyon'	21.5 ± 0.5	d	20	cd
group average	29.3 ± 2.3		60	
Small flowering group				
C. alpina	> 60		0	d
C. montana	> 60		0	d
C. orientalis	> 60		0	d
C. viticella	26.2 ± 2.4	c	90	ab
group average	26.2 ± 2.4		23	

1 results in the same column not sharing a common letter are significantly different ($\alpha = 0.05$).

Only three of the eleven varieties tested did not show any signs of wilt and these were all small flowered species, namely *C. alpina*, *C. montana* and *C. orientalis*. There was a significant effect of clematis group on both percentage of wilted plants after 60 days and average time interval before wilting ($\alpha = 0.05$). However, differences between varieties within the groups were often greater than those between the flowering groups.

©1999 Horticultural Development Council

Disease progression was especially quick in *C.* 'Ernest Markham' and in the small flowered *C. viticella*. The most resistant large flowered variety tested, *C.* 'Ville de Lyon', showed wilt twice early on in the experiment, but no additional disease after that. *C.* 'Comtesse de Bouchaud' was the last variety to start wilting, which did not occur until 37 days after inoculation. Most plants that wilted (65%) developed symptoms between 20 and 40 days after inoculation.

To investigate whether the susceptibility to wilt in practice was correlated to susceptibility to stem infection by *P. clematidina*, the varietal susceptibility to stem infection by the fungus was expressed as a susceptibility score:

S = ((fraction of wilted plants after 60 days x 10)/ the average number of days before wilting after 60 days) x 10.

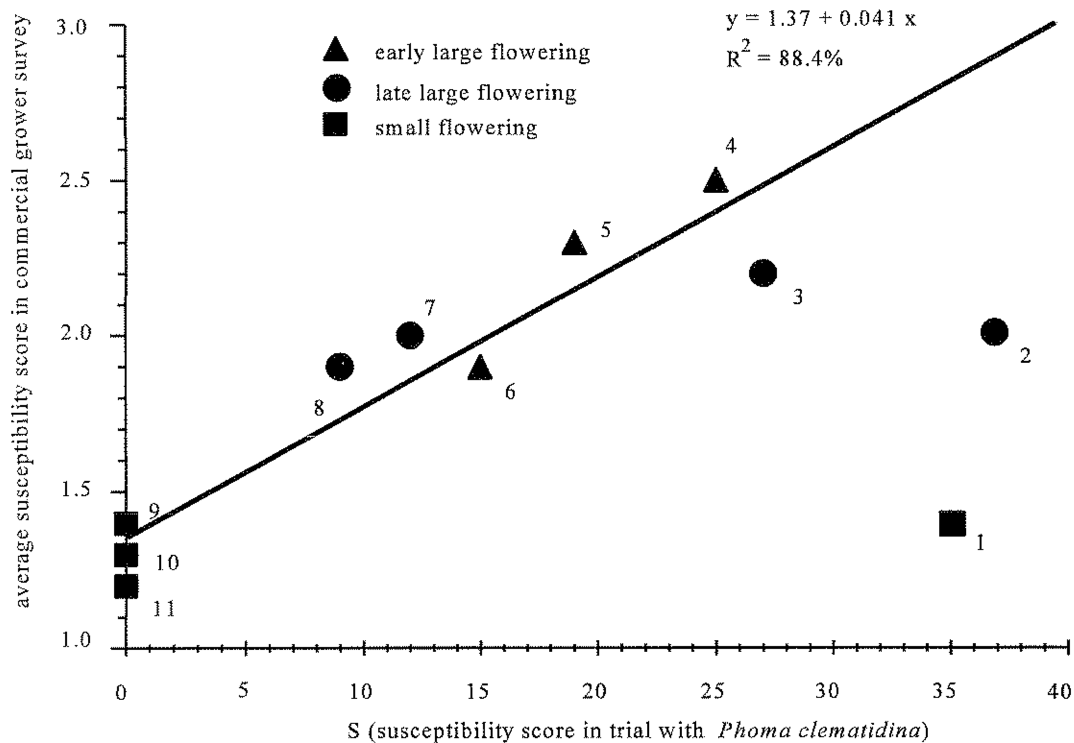
The higher the score, the more susceptible the variety. For varieties that did not wilt the score was taken as zero. In Figure 3, this score is set out against the average susceptibility score from the 1996 commercial grower survey (see Section 2). Most results indicated a positive linear relationship between the two scores. Only two varieties did not fit a linear relationship: *C. viticella* 'Purpurea Plena Elegans' and *C.* 'Ernest Markham', which is a *C. viticella*-type hybrid. These two varieties were less prone to wilt in practice than could be expected on basis of their susceptibility to *P. clematidina* in the variety trial. When the results from these two varieties were ignored, a significant linear relationship was found between the susceptibility of clematis varieties to *P. clematidina* in the stem inoculation trial and their susceptibility to wilt in practice according to the grower survey (see Figure 3) ($\alpha = 0.05$). The regression line for the scores in the amateur grower survey ($y = 1.13 + 0.057\,x$; $R^2 = 86.0\%$), which was also tested, did not differ greatly from the line for the commercial grower survey.

Discussion

Inoculation of unwounded internodal sections of stems on whole plants with spore suspension appeared to be the most reliable method to test the susceptibility of a range of clematis varieties to wilt caused by *P. clematidina*, and was therefore used in the variety trial. The use of spores represented the natural situation closely and it enabled the use of a mix of isolates differing in virulence.

For most clematis varieties, the results of the variety trial confirmed the results of the trials with *C.* 'Henryi' and *C. montana*, namely that susceptibility to wilt in practice is correlated to susceptibility to infection by *P. clematidina*. A positive linear relationship was found between the results of the stem inoculation trial with *P. clematidina* and the 1996 survey results for nine of the eleven varieties tested. This indicates that the inoculation of unwounded internodal stems with spores of a mix of *P. clematidina* isolates could be a simple and effective way to predict the susceptibility of new clematis varieties to wilt in practice. Only for *C. viticella* and closely related varieties this test would not be useful. Even though *C. viticella* and *C.* 'Ernest Markham' were the worst affected varieties in the stem inoculation trial, the 1996 survey showed that they are known as wilt resistant in practice. It could be that *C. viticella*- types are less susceptible to *P. clematidina* than the results of the variety trial suggested. The plants used in the trial had thin stems compared to most of the other varieties tested which could have influenced the results.

©1999 Horticultural Development Council



1 = *C. viticella*
2 = 'Ernest Markham'
3 = 'Lady Betty Balfour'
4 = 'Vyvyan Pennell'
5 = 'Henryi'
6 = 'Miss Bateman'
7 = 'Comtesse de Bouchaud'
8 = 'Ville de Lyon'
9 = *C. orientalis*
10 = *C. alpina*
11 = *C. montana*

Figure 3. **Relationship between S (susceptibility score in the stem inoculation trial with *Phoma clematidina* after 60 days) and average wilt susceptibility score in the 1996 commercial grower survey for different clematis varieties. The regression line is based on varieties 3 to 11.**

©1999 Horticultural Development Council

However, it is more likely that the general vigour of *C. viticella* and related varieties normally compensates for their susceptibility to *P. clematidina* and that they easily outgrow infection by the fungus if this occurs naturally. Results of trials by Ebben & Last (1966), who also found that internodal stems of *C. viticella* were susceptible to *P. clematidina*, and Smith (1987), who reported that leaves of *C.* 'Ernest Markham' were relatively susceptible to the fungus, support this idea.

The positive linear correlation between susceptibility to *P. clematidina* in the variety trial and susceptibility to wilt in practice appears to indicate that the fungus is a common cause of clematis wilt in practice. However, it cannot be excluded that varieties which are more susceptible to fungal infection, are also more susceptible to other causes of wilt such as mechanical damage. Unlike susceptibility to wilt in practice (Howells, 1994b), the susceptibility of clematis varieties to *P. clematidina* could not be easily traced back to their parentage. The results with *C. viticella* and *C.* 'Ernest Markham' showed, that *C. lanuginosa* as a parent does not play a decisive role in susceptibility to the fungus.

©1999 Horticultural Development Council

7. CHEMICAL CONTROL AND CULTURAL FACTORS

7.1 Benzimidazole resistance *in vitro*

Aim

To establish whether British isolates of *P. clematidina* show resistance against benzimidazole fungicides *in vitro*.

Methods

Mycelial plugs of six different isolates of *P. clematidina* were placed on V8-agar plates containing one of the following benzimidazole (-related) fungicides in active ingredient (a.i.) concentrations of 2, 10, 20 or 100 mg/l: benomyl (Benlate Fungicide by Dupont, 50% w/w), carbendazim (Bavistin DF by BASF, 50% w/w) or thiophanate-methyl (Mildothane Liquid by Hortichem, 500 g/l). Nine other isolates of *P. clematidina* were tested for resistance to carbendazim only. Agar plates were sealed with laboratory film and incubated at 20°C in constant darkness in a randomised block design with five blocks. The diameter of mycelial growth was measured after seven days and expressed as a percentage of the corresponding control which was grown on agar without fungicide. Other features such as sporulation and colony appearance were noted. Results were statistically analysed using ANOVA for overall effects and Duncan's Multiple Range test or Mann-Whitney U-tests for individual differences.

Results

The isolates used in this experiment belonged to three different morphological groups (see Chapter 4) and thus showed clear differences in both growth and sporulation on the medium without fungicide. As Figure 4 shows, four of the six isolates were resistant to the benzimidazole (-related) fungicides. They grew almost uninhibited in the presence of thiophanate-methyl in concentrations up to 100 mg/l. Carbendazim inhibited mycelial growth at 100 mg/l by up to 57%, but was much less effective at lower concentrations. Resistance against benomyl was weakest with a growth reduction of up to 44% at 20 mg/l and 79% at 100 mg/l. The differences between the three fungicides at 100 mg/l were significant for all resistant isolates ($\alpha = 0.05$). No significant differences in growth in presence of the different fungicides between the four resistant isolates or between the two morphological groups they belonged to were detected ($\alpha = 0.05$).

In the control, the benzimidazole resistant isolates were distinctly different in morphology from the sensitive isolates, but on the media with benzimidazole fungicide they often more closely resembled the benzimidazole sensitive isolates, forming less mycelium and chlamydospores, and more pycnidia with spore mass.

Mycelial growth of the two benzimidazole sensitive isolates was almost completely inhibited by carbendazim at all concentrations and by benomyl at concentrations higher than 2 mg/l.

©1999 Horticultural Development Council

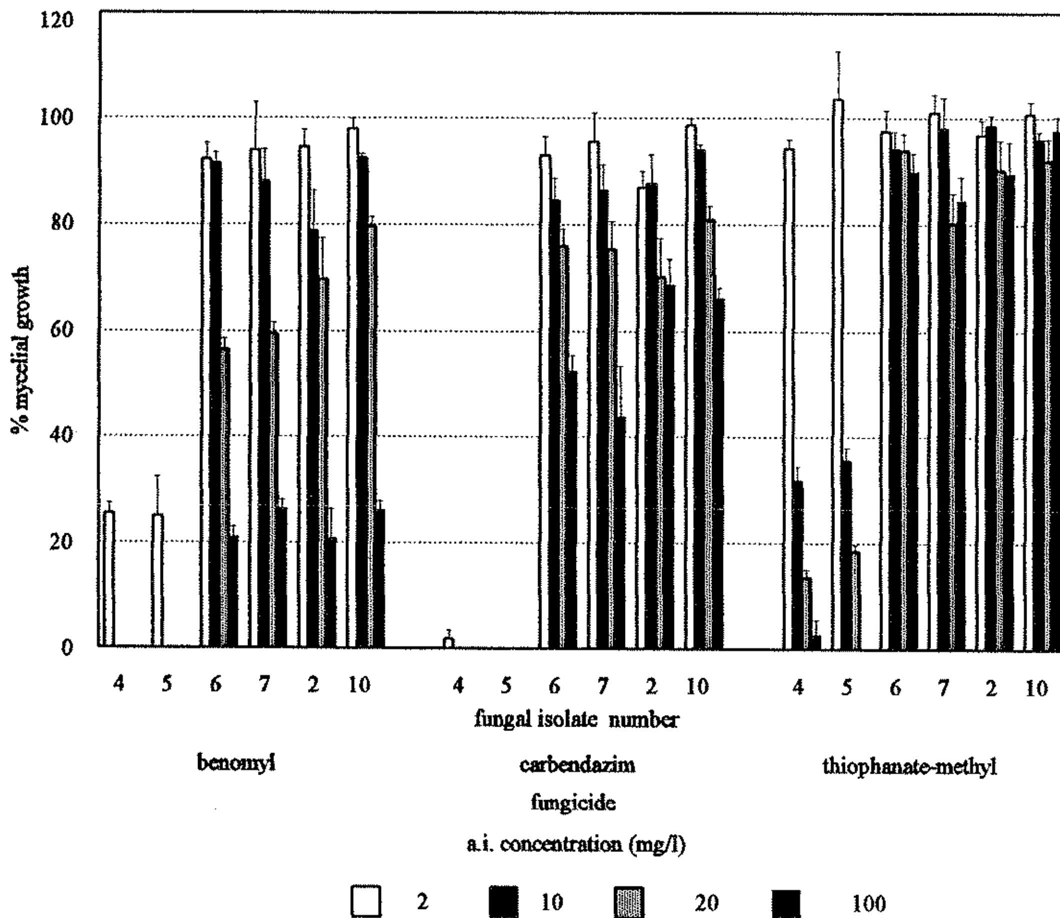


Figure 4. Average mycelial growth of six isolates of *Phoma clematidina* at 20°C after seven days on V8A amended with different benzimidazole (-related) fungicides at different concentrations, as a percentage of the control (n = 5) (S.E. bars). Isolates 4 & 5 are in morphological group III, 6 & 7 in group I and 2 & 10 in group II .

However, thiophanate-methyl was much less effective. At 2 mg/l, this fungicide had almost no inhibitive effect on fungal growth at all, while complete growth inhibition took place only at 100 mg/l. Nevertheless, the effect of thiophanate-methyl on the two sensitive isolates was significantly stronger at the three highest concentrations than its effect on the four resistant isolates ($\alpha = 0.05$).

Of the nine other isolates of *P. clematidina* tested, only one showed resistance to carbendazim at concentrations up to 100 mg/l.

©1999 Horticultural Development Council

Discussion

One third of the isolates of *P. clematidina* tested, showed no or only limited growth reduction in the presence of carbendazim in concentrations up to 100 mg/l and can therefore be indicated as resistant to this fungicide. The experimental results indicated that resistance to carbendazim is coupled to resistance to the related fungicides benomyl and thiophanate-methyl. The discovery of benzimidazole resistant isolates of *P. clematidina* in the UK was not unexpected since resistance had been reported in the Netherlands earlier (Van Kuik & Brachter, 1997). Whether the resistant strains found in the UK have developed independently under pressure of the widespread use of benzimidazole fungicides, in particular carbendazim and benomyl, or whether they were imported with infected Dutch clematis plants is unknown. The benzimidazole resistant isolates originated from different nurseries and private gardens around the UK and their incidence was high enough to partly explain the problems with chemical control of clematis wilt experienced on many British nurseries (see Section 2).

Although carbendazim and benomyl are thought to have the same mode of action, benomyl was clearly more effective against *P. clematidina in vitro* than carbendazim. This agrees with the general idea on the difference in effectiveness between the two fungicides in practice. It could be that benomyl has an additional mode of action which is lacking in carbendazim. One of the breakdown products of benomyl is known to inhibit cutinase production in fungi which may affect the infection process *in vivo* (Köller *et al.*, 1982), but this is unlikely to play a role *in vitro*. Another explanation could be that Benlate fungicide contains an ingredient, not present in Bavistin DF, which adds to or enhances the effect of benomyl.

Although colony morphology *in vitro* on agar without fungicide indicated two distinctive groups of benzimidazole resistant isolates of *P. clematidina* (see Section 4), no differences in reaction to benzimidazole fungicides were observed between the two morphological groups, which suggests that their basis of resistance is similar.

7.2 Evaluation of fungicides *in vitro*

Aim

To evaluate different fungicides for their effect on mycelial growth and spore germination in *P. clematidina in vitro*.

Methods

The effect on mycelial growth and spore germination of 15 fungicides listed in Table 5 were tested using a benzimidazole resistant isolate of *P. clematidina*. The fungicides were selected on the basis of indications of their effectiveness against *Phoma* and related fungi in the literature. Mycelial growth was tested as described in Section 7.1. Spore germination was tested by making a spore suspension in water of the isolate in question, putting drops of the spore suspension on clean glass slides and mixing them with drops of fungicide, so that the final concentrations of active ingredient were 2, 10, 20 and 100 mg/l, and the final spore concentration 10^5 spores/ml.

©1999 Horticultural Development Council

The drops were covered with a cover slip and incubated in sealed plastic Petri-dishes containing moist filter paper at 20°C in constant darkness in a randomised block design with four blocks. Observations were made at t = 24 and t = 48 h, counting 50 spores per replicate. Only spores with germ tubes longer than 5 μm were considered to have germinated. The number of germinated spores was expressed as a percentage of the corresponding control which contained water only. Results were statistically analysed using ANOVA.

Table 5. Fungicides tested against *Phoma clematidina in vitro*.

fungicide group/ active ingredient (a.i.) (abbreviation)	product name (experimental code)	manufacturer	a.i. concentration
Systemic			
benzimidazole (-related)			
benomyl (ben)	Benlate Fungicide	Dupont	50% w/w
carbendazim (car)	Bavistin DF	BASF	50% w/w
thiophanate-methyl (thi)	Mildothane Liquid	Hortichem	500 g/l
imidazole			
imazalil (ima)	Fungaflor	Hortichem	200 g/l
prochloraz (prc)	Octave	AgrEvo	46% w/w
morpholine			
fenpropimorph (fen)	Corbel	BASF	750 g/l
triazole			
difenoconazole (dif)	Plover	Novartis	250 g/l
propiconazole (prp)	Tilt 250 EC	Ciba Agric.	250 g/l
tebuconazole (teb)	Folicur	Bayer	250 g/l
Protective			
anilinopyrimidine			
pyrimethanil (pyr)	Scala	Promark	400 g/l
aromatic			
chlorothalonil (chl)	Bravo 500	BASF	500 g/l
tolylfluanid (tol)	Eupareen (UK 456)	Bayer	50% w/w
copper			
copper ammonium carbonate (cop)	Croptex Fungex	Hortichem	82 g/l
strobilurin			
azoxystrobin (azo)	Amistar (YF9247)	Zeneca	250 g/l
kresoxim-methyl (kre)	Stroby (BAS 49002F)	BASF	50% w/w

©1999 Horticultural Development Council

Results & Discussion

There was a significant effect of fungicide on both mycelial growth and spore germination of *P. clematidina* ($\alpha = 0.05$). There were important differences between and within the groups of benzimidazole, other systemic and protective fungicides, which is clearly shown by the EC_{50} (effective concentration at which 50% inhibition takes place) values given in Table 6. The isolate of *P. clematidina* used in this experiment was resistant to the three benzimidazole fungicides tested in the experiment described in Section 7.1, and this benzimidazole resistance was confirmed in the present experiment. Carbendazim had almost no effect on either mycelial growth or spore germination in comparison to the control. However, as the EC_{50} values indicate, benomyl was much more effective especially in preventing spore germination. Most of the non-benzimidazole systemic fungicides inhibited mycelial growth at low concentrations, although tebuconazole was slightly less effective. Only imazalil, propiconazole and tebuconazole inhibited spore germination completely at 100 mg/l. The effect on spore germination was very similar within this group except for difenoconazole which was overall the most effective systemic fungicide tested.

The protective fungicides had more or less the opposite effect of the non-benzimidazole systemic fungicides and were better in reducing spore germination than mycelial growth. The copper fungicide even considerably enhanced mycelial growth. The difference in EC_{50} for mycelial growth between azoxystrobin and kresoxim-methyl in Table 6 is deceptive, since their inhibitive effect at 2 mg/l was almost similar (around 50%) and for both fungicides an increase in concentration hardly enhanced their effectiveness. However, the strobilurins, as well as chlorothalonil, were especially effective against spore germination, since with these three fungicides even the swelling, which is the first stage in the germination process, had not started after 48 h in most of the fungal spores examined. Pyrimethanil and tolylfluanid had the most inhibitive effect on mycelial growth, comparable to that of the non-benzimidazole systemic fungicides.

These results show that there might be several fungicides which are more effective against *P. clematidina* than carbendazim and prochloraz, which are widely used by clematis growers at present. Difenoconazole (Plover), pyrimethanil (Scala) and the two strobilurins (Amistar and Stroby) are especially promising since they inhibit both mycelial growth and spore germination very effectively. However, trials on whole plants are needed to complement their assessment as effective chemicals for control of *P. clematidina*.

©1999 Horticultural Development Council

Table 6. EC_{50} values (mg/l) for different fungicides against mycelial growth and spore germination of *Phoma clematidina* (benzimidazole resistant isolate) *in vitro* (n = 4).

fungicide	EC_{50} (mg/l)	
	mycelial growth	spore germination
benomyl (Benlate)	63	16
carbendazim (Bavistin DF)	10^8	-**
thiophanate-methyl (Mildothane liquid)	200	41
difenoconazole (Plover)	< 2	< 2
fenpropimorph (Corbel)	< 2	20
imazalil (Fungaflor)	< 2	36
prochloraz (Octave)	< 2	50
propiconazole (Tilt 250 EC)	< 2	3
tebuconazole (Folicur)	2	10
azoxystrobin (Amistar)	2	< 2
chlorothalonil (Bravo 500)	71	< 2
copper (Croptex Fungex)	-*	7
kresoxim-methyl (Stroby)	77	< 2
pyrimethanil (Scala)	< 2	< 2
tolylfluanid (Eupareen)	2	4

* the copper fungicide promoted mycelial growth rather than inhibit it; ** carbendazim did not inhibit spore germination at all.

7.3 Evaluation of fungicides *in vivo*

Aim

To evaluate different fungicides for their effect on infection of clematis plants by *P. clematidina in vivo.*

Methods

Six fungicides were selected from the 15 mentioned in Table 6 on basis of their effect on mycelial growth and spore germination *in vitro* as described in Section 7.2. Carbendazim (Bavistin DF, 1 g/l), prochloraz (Octave, 1 g/l), difenoconazole (Plover, 1 g/l), pyrimethanil (Scala, 1 ml/l), azoxystrobin (Amistar, 1 ml/l) and kresoxim-methyl (Stroby, 1 g/l) were sprayed fortnightly on groups of nine six months old *C.* 'Henryi' plants kept in a randomised block design with four replicates in a ventilated polytunnel. Controls were sprayed with tap water instead of fungicide. All fungicides were sprayed in the concentrations indicated using a knapsack sprayer with a flat, single nozzle lance at a pressure of 3 bar for 6 seconds per group of nine plants.

The plants were inoculated with spores of *P. clematidina* two days after the first spray, and again two days after the second spray.

©1999 Horticultural Development Council

Each plant in half of the groups was sprayed with 5 to 10 ml (depending on the size of the plant) of a spore suspension (10^6 spores/ml) of a benzimidazole sensitive isolate of *P. clematidina*, and each plant in the remaining groups with spores of a benzimidazole resistant isolate. One group per block served as a double control (no fungicide, no inoculum) and was sprayed with water. The plants were watered overhead twice a day, except for the first two days after each fungicide spray. For four months, each plant was checked for the presence of *Phoma* leaf spots, wilted stems or phytotoxic effects every two to three weeks. Results were statistically analysed using ANOVA for overall effects and Duncan's Multiple Range test for individual differences.

Results

There was a significant effect of fungicide treatment on the average number of leaf spots per plant group after 12 weeks ($\alpha = 0.05$). Disease was most severe in the inoculated control groups, but the double control (no inoculation, no fungicide) showed only two leaf spots after 12 weeks and was otherwise healthy.

Figure 5 shows for both the benzimidazole resistant and sensitive isolate of *P. clematidina*, that most fungicides reduced the number of leaf spots in comparison to the control. A significant effect of *P. clematidina* isolate on number of leaf spots could not be detected ($\alpha = 0.05$). Most fungicides gave similar results for both the benzimidazole resistant and sensitive isolate and only carbendazim was considerably less effective against the benzimidazole resistant isolate than against the sensitive isolate. After 12 weeks, there was only a slight difference between the control and carbendazim for the plants inoculated with the benzimidazole resistant isolate. However, carbendazim was effective against the benzimidazole sensitive isolate (see Figure 5). Table 7 shows that difenoconazole and the two strobilurin fungicides were overall the most effective fungicides tested, and that no significant difference with the double control could be detected for these fungicides ($\alpha = 0.05$). Pyrimethanil was overall the worst performing fungicide tested and in addition had a phytotoxic effect and caused small white lesions on the leaves of some of the clematis plants treated. Prochloraz did reduce the number of leaf spots significantly compared to the control, but was not as effective as difenoconazole and the strobilurins ($\alpha = 0.05$).

After 16 weeks, 18 plants had wilted (3%), most of which were in the inoculated control (2%). One third of wilted plants had been inoculated with the benzimidazole resistant isolate of *P. clematidina*, two thirds with the benzimidazole sensitive isolate. None of the plants in the double control wilted during the trial. As Table 7 shows, all fungicide treatments reduced the incidence of wilt. Least effective against wilt were pyrimethanil and prochloraz, while no wilt occurred in plants treated with kresoxim-methyl or difenoconazole.

©1999 Horticultural Development Council

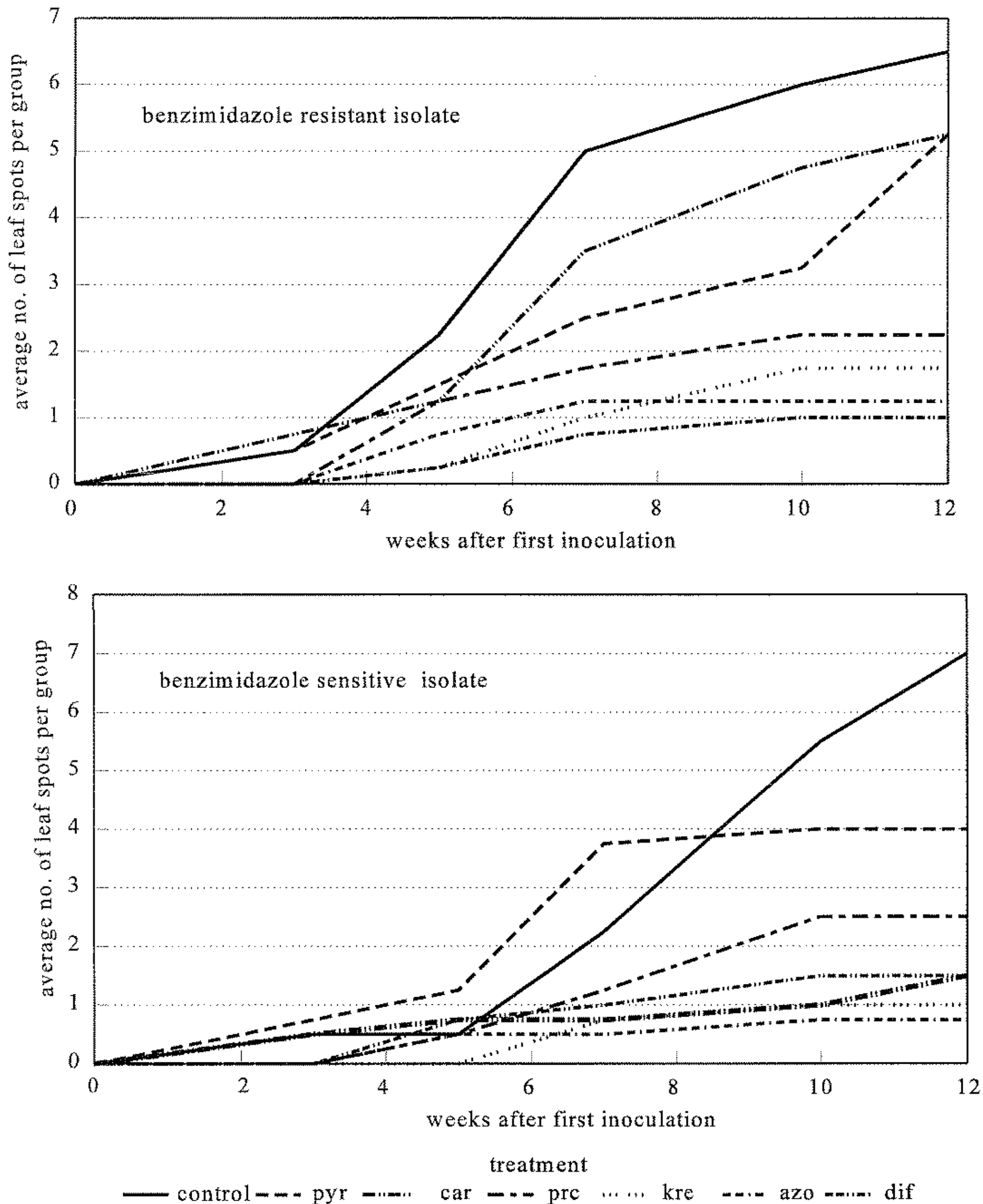


Figure 5. **Average number of *Phoma* leaf spots per group of nine *Clematis* 'Henryi' plants in time after inoculation with spores of a benzimidazole resistant (top) or sensitive (bottom) isolate of *Phoma clematidina* and fortnightly treatment with different fungicides in a polytunnel (n = 4). For abbreviations of fungicide names see Table 5.**

©1999 Horticultural Development Council

Table 7. Effect of different fungicide treatments on average number of leaf spots (12 weeks after the first inoculation) and on average number of wilted plants (16 weeks) within groups of nine *Clematis* 'Henryi' plants after inoculation with spores of *Phoma clematidina* (n = 4).

fungicide treatment	average number of leaf spots ± S.E.[1]	average number of wilted plants
inoculated control	6.75 ± 2.11 a	2.5
carbendazim	3.38 ± 0.90 b	0.3
difenoconazole	1.25 ± 0.75 c	0
prochloraz	2.38 ± 0.55 d	0.8
azoxystrobin	1.00 ± 0.35 c	0.3
kresoxim-methyl	1.38 ± 0.69 c	0
pyrimethanil	4.63 ± 0.47 e	0.8
double control	0.50 ± 0.29 c	0

1 results in the same column not sharing a common letter are significantly different ($\alpha = 0.05$).

Discussion

Pyrimethanil was very effective against *P. clematidina in vitro*. Initial tests had shown that it could also be effective against the fungus on clematis *in vivo*, but had phytotoxic side effects (see Annual Report Year 2). Therefore, the rate in the present trial was halved. Yet, phytotoxic symptoms still appeared directly after the first application while the effectiveness of pyrimethanil was largely lost, which rules this fungicide out as a candidate for use against clematis wilt in practice.

The results of this fungicide trial showed further that carbendazim can still be effective against benzimidazole sensitive isolates of the fungus, but that if resistant isolates are present, difenoconazole, azoxystrobin or kresoxim-methyl might be better choices. Unfortunately, these fungicides are presently not allowed for use on clematis. At the moment, the strobilurins can be used only on certain field crops, but difenoconazole has already been approved for use on protected ornamentals (Whitehead, 1999).

©1999 Horticultural Development Council

7.4 Effect of watering method on disease spread and control

Aim

To study the effect of watering method on spread and control of disease caused by *P. clematidina*

Methods

A ventilated polytunnel was divided in two parts by putting up a plastic screen in the middle (side ways). Groups of 25 six months old *C.* 'Henryi' plants were placed in a randomised block design with four replicates on each side of the screen. Plants on one side of the polytunnel were watered overhead by sprinklers, the others by individual drip irrigation. Plants infected with *P. clematidina* and showing leaf spot and wilt symptoms were placed in the middle of half of the groups. The infected plants had been inoculated two weeks earlier with a mix of spores of two isolates of *P. clematidina* (both benzimidazole sensitive). Healthy, non-inoculated plants were placed in the middle of the remaining groups as a control. Half of the inoculated groups and half of the control groups on each side of the polytunnel were sprayed fortnightly, alternately with carbendazim (Bavistin DF, 1 g/l) and prochloraz (Octave, 1 g/l). The rest of the groups were sprayed with tap water. The middle plant of each group was always removed during spraying. Plants were watered three times a day except for the first two days after each fungicide spray. For three months, each plant was checked for the presence of leaf spots or wilted stems every two to three weeks, and the pattern of disease spread within each group was noted. Results were statistically analysed using ANOVA for overall effects and Duncan's Multiple Range test for individual differences.

Results

There was a clear effect of watering method on the spread of disease caused by *P. clematidina* within the groups of plants in time. Due to the fact that the two watering treatments had to be separated in space for obvious reasons and could not be integrated, the differences could not be statistically tested. However, as Figures 6 and 7 show, disease spread much more quickly in the groups which were watered overhead than in those which were drip irrigated and after 12 weeks, the average number of leaf spots per plant was at least five times higher for plants with overhead watering than for plants in the section with drip irrigation (see Table 8).

Figures 6 and 7 further indicate that the course of disease development in time was similar for the control plants and the fungicide treated plants. In the section with overhead watering after 12 weeks, the average number of leaf spots per plant was significantly lower for the fungicide treated groups than for the control plants, but only for the plants with inoculum ($\alpha = 0.05$) (see Table 8). Most leaf spots in this section appeared in the lower part of the plants where the leaves were growing close together and humidity was easily maintained and no leaf spots were found higher than 50 cm from soil level.

©1999 Horticultural Development Council

Table 8. Effect of watering method and fungicide treatment on average number of leaf spots per plant within square groups of 25 *Clematis* 'Henryi' plants with a *Phoma clematidina* infected plant in the middle (n = 4).

watering method	treatment		average number of leaf spots per plant ± S.E.[1]
drip irrigation	control	with inoculum	
		inner ring	0.063 ± 0.036 a
		outer ring	0.16 ± 0.054 a
	fungicide	with inoculum	
		inner ring	0.094 ± 0.060 a
		outer ring	0.16 ± 0.040 a
overhead watering	control	with inoculum	
		inner ring	2.19 ± 0.53 a
		outer ring	0.91 ± 0.41 a
	fungicide	with inoculum	
		inner ring	0.69 ± 0.19 a
		outer ring	0.50 ± 0.081 b

1 results in the same column not sharing a common letter are significantly different ($\alpha = 0.05$).

After 12 weeks, significantly more spots had formed on plants in the inner ring of groups than on plants further removed from the source of inoculum, but individual differences could not be detected in case of the control ($\alpha = 0.05$) (see Table 8). However, spots were regularly observed on leaves on the outside of groups as well and the course of disease development in time was similar for the inner and outer rings of groups watered overhead (see Figures 6 and 7). In the groups watered overhead, disease increased at an almost constant rate when an infected plant was present in the middle and did not shows any signs of levelling off in time.

In the drip irrigated groups, the total number of leaf spots was very low and mostly appeared on plants in the outer ring of groups (see Table 8) and higher up than in plants which were watered overhead.

©1999 Horticultural Development Council

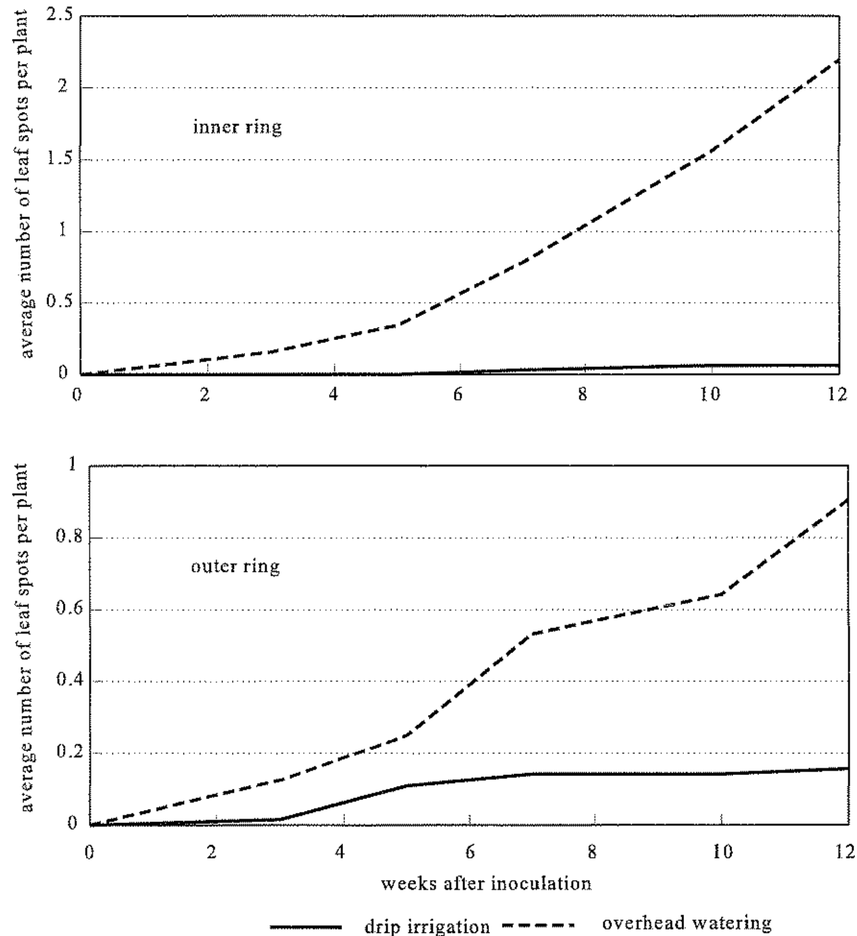


Figure 6. Average number of leaf spots per plant in the inner (top) and outer (bottom) ring in square groups of 25 *Clematis* 'Henryi' plants, not treated with fungicide and with a *Phoma clematidina* infected plant in the middle, for two different watering methods in time (n = 4).

The leaf spots were usually associated with earwig damage to leaves or occasionally with the presence of earwigs themselves, and it seems therefore likely that these insects were responsible for the spread of disease within the crop. This would also explain the few spots which were found in the groups which had healthy plants in their middle. No significant difference between the fungicide treated and non-treated plants or between the inner and outer ring in the drip irrigated groups could be detected ($\alpha = 0.05$).

Only two wilted shoots were observed during the experiment. These were in the inner ring of one group watered overhead, not treated with fungicide and with an infected plant in the middle. Both wilted plants showed leaf spots as well.

©1999 Horticultural Development Council

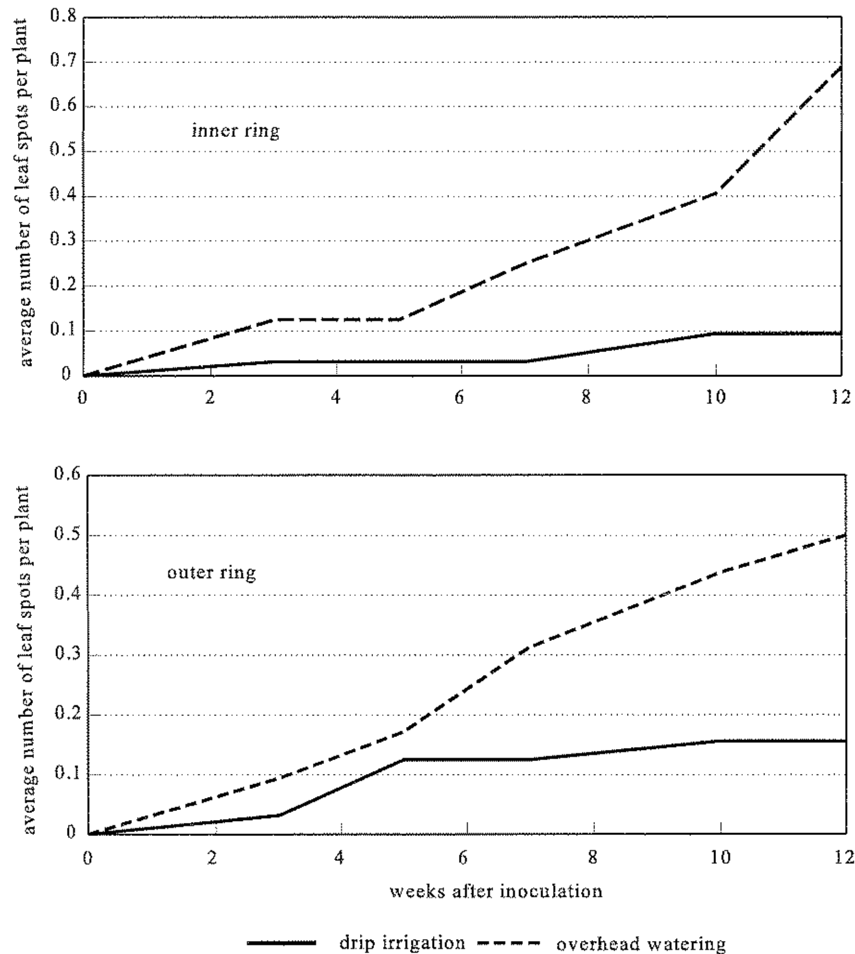


Figure 7. Average number of leaf spots per plant in the inner (top) and outer (bottom) ring in square groups of 25 *Clematis* 'Henryi' plants, treated fortnightly with fungicide and with a *Phoma clematidina* infected plant in the middle, for two different watering methods in time (n = 4).

Discussion

As with other *Phoma* species, water splash is clearly an important factor in the spread of *P. clematidina* from plant to plant, as the watering trial showed. In comparison with overhead irrigation, drip irrigation severely restricted disease development within groups of clematis plants, even when a *P. clematidina* infected plant was close by. This was probably due to two factors: firstly the absence of spread of the spores by water splash, and secondly the absence of moisture on any of the plant parts apart from the roots which prevented any spores that did spread from germinating and initiating infection. The trial showed that a single infected clematis plant may form a source of infection for a whole batch of plants which are initially healthy. Although spores appeared to be splashed over only short distances, the fungus spread quickly and reached the edge of the groups within three months after its introduction.

©1999 Horticultural Development Council

The fungicide treatment significantly reduced the severity of the symptoms per plant, but failed to prevent spread of the fungus to healthy plants in many cases. With drip irrigation, the fungicide application was irrelevant. Thus, the results of the watering trial suggest that the introduction of drip irrigation or another watering method which moistens only the plant roots might be more effective in reducing the incidence of clematis wilt than the application of fungicides.

The limited spread of *P. clematidina* by earwigs observed in the watering trial is in agreement with findings by Renfro & Wilcoxson (1963) on spread of *P. medicaginis* by a range of insects. However, the extensive feeding damage that earwigs cause to clematis flowers and leaves is probably more important to growers than their role as occasional vectors of *P. clematidina.*

7.5 Effect of growing medium on root infection

Aim

To investigate whether plant growing medium influenced root infection of clematis by *P. clematidina*

Methods

Three months old *C.* 'Henryi' plants were potted up in May in two litre plastic containers using either 100% Irish moss peat (pH 4.5), a mix of peat and composted pine bark (75:25) (pH 5.1), a mix of peat and perlite (75:25) (pH 4.7), or a mix of peat and potting grit (90:10) (pH 4.6). A mix of Osmocote Plus (4 g/l) and magnesian limestone (1.5 g/l) was added as fertiliser. Half of the containers were inoculated with six mycelial plugs each of two different isolates of *P. clematidina* . Sterile agar plugs were used for the remainder as a control. The plugs were placed about 20 cm from the bottom of the container, so that the clematis roots would come into contact with them after growing into the medium. Plants were kept in a randomised block design with ten replicates in a ventilated polytunnel, shaded by green netting, and watered daily so that the growing medium was constantly moist. Plants were regularly inspected for wilting symptoms, and lifted after 100 days to check for root rot symptoms. The above ground plant parts of the controls were harvested and dried for 20 hours at 100°C in an oven to establish dry weight. Results were statistically analysed using ANOVA and χ^2-tests.

Results and Discussion

None of the clematis plants wilted during the course of the growing medium experiment and most grew well and flowered. Only a few plants in the peat/bark mix lacked vigour and showed a yellow/red discolouration of the leaves. No extensive root rot was found in any of the treatments, but half of the inoculated plants showed black rot at root tips, which resembled root rot symptoms caused by *P. clematidina* found earlier in inoculated plants in water culture (see Section 6). Upon microscopical examination, some of the lesions showed the presence of *Phoma* pycnidia. Some of the black root tips plated out on V8A yielded *P. clematidina,* while this fungus was not found in any of the controls.

©1999 Horticultural Development Council

No significant effect of growing medium on disease incidence or dry weight of the control (see Table 9) was detected ($\alpha = 0.05$).

Table 9. Effect of growing medium on percentage of inoculated *Clematis* 'Henryi' plants with *Phoma clematidina* root rot and average dry weight (g) of the above ground parts of the control plants (n = 10).

growing medium	% infection	average dry weight (g) of the control ± S.E.
100% peat	40	20.5 ± 1.4
75% peat/25% bark	30	14.4 ± 2.8
75% peat/25% perlite	60	16.2 ± 0.8
90% peat/10% grit	70	16.1 ± 1.3

Although extensive root infection by *P. clematidina* was regularly observed in clematis plants from nurseries (see Section 3) and the fungus was shown to be capable of causing such symptoms in healthy plants (see Section 6), symptoms in the growing medium experiment were not severe. This could have been due to the amount of inoculum or the duration and conditions of the experiment. Stressed plants are usually more susceptible to fungal attack, but although the moisture in the media was kept high continuously to facilitate infection, this did not appear to put the plants in a stressful situation. Many root systems were surprisingly healthy and apparently no important root pathogens other than *P. clematidina* profited from the moist soil conditions.

Root infection by *P. clematidina* took place without causing any above ground symptoms and although root infection is generally not as devastating as stem infection, epidemiologically it could nevertheless be important. Growers will treat clematis plants with limited root infection by *P. clematidina* as if they were healthy and in this way the fungus might spread to other nurseries, private gardens and plant collections without detection.

7.6 Saprophytic growth on plant material

Aim

To study the growth and survival of *P. clematidina* on dead plant debris.

Methods

Leaves of *C.* 'Henryi', annual meadow grass (*Poa annua* L.), and leaves and stems of common chickweed (*Stellaria media* L.) were collected from living plants and autoclaved. The dead plant material was then divided into plastic Petri dishes.

©1999 Horticultural Development Council

Five dishes per treatment were inoculated with V8A- plugs of a *P. clematidina* isolate. Another five were inoculated with sterile agar plugs as a control. All plates were sealed with laboratory film and incubated at 20°C in constant darkness. After five weeks the plant material was assessed visually for the presence of fungal mycelium and pycnidia and material which had not been in direct contact with the inoculum, was plated out on V8A to check for the presence of *P. clematidina.*

In addition, leaves of *C.* 'Henryi' were moistened with water and autoclaved in glass jars closed with metal lids. Three V8A-plugs with a *P. clematidina* isolate were added to each of five jars. Sterile agar plugs were added to the remaining five jars as a control. The jar lids were sealed with tape and the jars placed outside on a balcony in Derby in October. Five months later, the jars were recovered, opened and the leaf material inside was assessed as described above.

Results and Discussion

Phoma-like mycelium and pycnidia were observed on all inoculated plant material up to several centimetres from the inoculation point. The fungus had grown into debris of clematis, grass as well as chickweed, but most pycnidia were found on dried grass. When plated out, all replicates of clematis and grass yielded *P. clematidina,* but the fungus was recovered from only one piece of chickweed. *P. clematidina* also invaded all clematis leaf material kept outside during the winter. Most leaves were covered with pycnidia, but these did not have the normal globose shape. Instead, they each had formed several long ostioles (100 - 300 µm in length), which, however, exuded normally shaped and sized conidia. These pycnidial structures resembled those formed on agar plates after prolonged incubation *in vitro*. When plated out on V8A the pycnidial structures yielded normal colonies of *P. clematidina*. No sexual fruiting bodies were observed on the plant material, despite the long incubation under outside conditions. In neither experiment was *P. clematidina* isolated from the non-inoculated controls or were other fungi recovered from any of the plant material tested.

The fact that *P. clematidina* grew saprophytically on dead clematis material agrees with results of experiments carried out by Gloyer (1915). However, the fact that it also grew and reproduced on material of other plants is new and means that growers need to take even stricter hygienic measures to prevent the spread and survival of this fungus. In the present study, all plant material used had been autoclaved which killed any other saprophytic organisms which might normally compete with *P. clematidina* and restrict its growth, but the amount of pycnidia formed on the dead material was considerable and showed that the fungus is well adapted to an existence without living clematis plants. The weed species used are two very common ones, but it is likely that dead material of other plant species might also serve as a substrate for growth of *P. clematidina*. The pycnidial structures observed on dead clematis material have not been reported for *P. clematidina* before, but do slightly resemble pycnidia with long ostioles formed by some other *Phoma* species. These pycnidia are atypical of *P. clematidina* and might have developed in reaction to certain environmental conditions (De Gruyter, pers. comm.). There were no indications that they were in any way associated with the sexual stage of the fungus, which appears to be very rare and remains to be reported from cultivated clematis plants.

©1999 Horticultural Development Council

8. OVERALL DISCUSSION

Both the 1996 grower surveys and the visits to several British nurseries showed that clematis wilt is an important and widespread problem on nurseries around the UK. Losses, for as far as they could be established, were often considerable and many growers experienced problems with disease prevention and control. Occasionally, this even forced growers to abandon the cultivation of large flowered varieties which were generally considered to be highly susceptible to wilt.

Fungal isolations from plants collected from both nurseries and private gardens yielded several fungal species which had been mentioned in the literature as possible causal agents of clematis wilt. However, in pathogenicity trials only one of the species tested was shown to be capable of causing the wilting symptoms so often observed in practice, and this was *Phoma clematidina*. It was the fungal species most commonly isolated from diseased plants. The formation of chlamydospores, which were formed by all isolates of the fungus, facilitated identification and prevented confusion with closely related species of *Phoma*, which were occasionally recovered from diseased clematis as well. *P. clematidina* had already been suspected as an important cause of clematis wilt by earlier workers such as Gloyer (1915), Ebben & Last (1966) and Smith (1987), and although the average percentage recovery from plant material in the present studies was relatively low, the possibility of its important role in clematis wilt was supported by the results of several pathogenicity trials.

The fungus was confirmed as a cause of leaf spot as indicated earlier particularly by Smith (1987). However, inoculation of unwounded stems resulted much more quickly and regularly in wilt than leaf inoculation. In addition, *P. clematidina* was shown to be a cause of root rot in clematis and was not infrequently recovered from the roots of wilted nursery plants, which is important new knowledge especially in relation to the practice of cutting wilted plants down to soil level in the hope of re-growth. The fungus is dependent on moisture for spore germination and infection. The lower regions of the plant are normally those which stay moist for long periods of time and this may be why stem infection often occurs at soil level. Unfortunately, this is also the most damaging site for infection to occur since it usually results in death of almost the whole plant.

A clear positive correlation was found between the susceptibility of many different clematis varieties to stem infection by *P. clematidina* and their susceptibility to wilt in practice. This indirectly showed the importance of the fungus as a common cause of clematis wilt, which was further strengthened by the fact that *C. montana,* which is very resistant to wilt in practice, was found to be highly resistant to leaf- and stem infection by *P. clematidina*.

Microscopic studies of the events taking place on the surface of two clematis varieties differing in resistance to *P. clematidina*, suggested that disease resistance is based on defence mechanisms which may take effect as soon as the fungus makes contact with the plant surface.

©1999 Horticultural Development Council

The results from the studies on the pre-penetration stages of infection of clematis by *P. clematidina* complemented those on the post-penetration events carried out by Smith (1987). Together they show that *C. montana* is capable of blocking the fungus from further invasion at several stages of the host-pathogen interaction.

It is likely that the defence mechanisms found in *C. montana* are also the basis for resistance in other small flowered clematis species. However, both the fungal isolations and the disease trials showed that small flowered clematis are not immune to *P. clematidina* and that infection may occasionally occur. *C. viticella* and related varieties were shown to be especially susceptible to the fungus while in practice they are usually resistant to wilt. This suggests that these plants may be capable of harbouring the fungus without showing extensive symptoms, an idea that has also been put forward by Van Kuik (1999). Whether *P. clematidina* may be present in clematis plants without causing any visible symptoms at all, as suspected by Ebben & Last (1966) and Wolff (1996), remains unclear. Presently, a PCR-based detection method for *P. clematidina* is being developed in the Netherlands, which, once available, should be able to clarify the issue of latent infection (Van Kuik, pers. comm.). The detection method would also be helpful in determining the effectiveness of current isolation methods, since it might well be that the percentage recovery of *P. clematidina* from diseased material is much lower than its actual incidence.

The stem inoculation method developed in the present studies might form a useful tool to test newly bred clematis varieties on their resistance to wilt in practice. If a variety is found to be resistant to stem infection by *P. clematidina* using this test, it is very likely to be resistant to wilt in practice as well. If a variety is found to be susceptible to the fungus, it is important to determine the plant's parentage, since varieties close to *C. viticella* might still be vigourous enough to outgrow naturally occurring infection in most cases.

One of the most effective measures against clematis wilt that could be taken by growers and breeders would be to abolish the use and cultivation of large flowered varieties and to concentrate on the wilt resistant small flowered species. However, despite the problems with clematis wilt, large flowered varieties remain very popular with the general public and their cultivation therefore economically attractive. Thus, growers will need to take other measures to prevent the occurrence of wilt both on the nursery, and perhaps even more importantly, after purchase in the garden. Although the 1996 survey results and observations made on nurseries and during the trials suggested that mechanical damage due to handling or the wind may occasionally be a cause of wilt in clematis, it is now clear that *P. clematidina* is the most important cause. Thus, prevention and control of clematis wilt should be based mainly on prevention and control of this fungus.

The results of the commercial grower survey revealed that many growers believe the products currently available against clematis wilt are ineffective. Those growers who did apply chemicals mentioned a wide range of fungicides, the use of some of which appears to be based purely on a trial and error regime of application. The findings in the current project can partly explain the problems with chemical control observed in practice. Carbendazim and prochloraz, the two fungicides most commonly used by clematis growers against wilt, were found to be only partly effective against *P. clematidina*.

©1999 Horticultural Development Council

The occurrence of benzimidazole resistant isolates of the fungus means that carbendazim is ineffective against a considerable percentage of *P. clematidina* strains. However, it was shown that more effective fungicides against *P. clematidina* are potentially available and that difenoconazole and the strobilurins are especially promising. Yet, care needs to be taken to alternate several fungicides with different modes of action to prevent a repeat of the emergence of fungicide resistance.

Despite the fact that nursery hygiene has been known to be an important factor in the prevention of disease caused by *P. clematidina* ever since the publication of Gloyer's (1915) work, it became clear from the 1996 survey and the nursery visits that on many nurseries improvements could be made in this area. Simple measures such as the regular removal of diseased plants and plant debris, abolishment of the practice of cutting down wilted plants to soil level and growing them on, and disinfection of pruning tools between batches of plants will help to prevent survival and spread of *P. clematidina.*

Almost all clematis growers responding to the 1996 survey used overhead watering in their clematis crop. The watering trial in the current project clearly showed that this method is an elementary factor in spread of disease caused by *P. clematidina.* A change to a watering method in which only the roots of the plants are moistened is likely to reduce disease incidence to a level at which chemical applications against clematis wilt could be significantly reduced or even abolished, especially if combined with rigorous hygiene practices. In view of the current concerns for the environment, this might be preferable to a change in fungicide regime involving an on going application of a range of chemicals to clematis plants. By reducing the amount of moisture available to *P. clematidina* in the clematis crop, the survival of the fungus would be greatly reduced. However, for many growers the costs involved in changing the watering system may form an important obstacle.

The introduction of *P. clematidina* into private gardens and plant collections could be largely prevented if commercial growers and proprietors of garden centres would apply strict hygiene regimes and destroy diseased plants much more quickly than is presently the case. Horticultural consultants could play an important role by informing growers on this issue. However, in the end it is down to each clematis grower individually to take the measures necessary to bring about a significant reduction in the incidence of clematis wilt in the UK.

©1999 Horticultural Development Council

9. CONCLUSIONS

- Clematis wilt is a widespread and common disease both on nurseries and in private gardens around the UK.

- *Phoma clematidina* is the most common causal organism of clematis wilt in the UK.

- Large flowered clematis varieties are most susceptible to clematis wilt caused by *P. clematidina*. Small flowered species are mostly resistant though not immune. Resistance is based on a complex of mechanisms including papilla deposition in reaction to fungal penetration.

- *P. clematidina* may infect leaves, stems and roots of clematis. Direct infection of stems results in wilt most quickly and is a good indicator of resistance to wilt in practice for most clematis varieties.

- *P. clematidina* is well adapted to a saprophytic life style and may survive and reproduce on dead material of different plant species. An improvement in hygiene measures is possible on many British nurseries and is important in reducing disease incidence.

- Spores of *P. clematidina* are mainly spread by water splash and a change in watering method from overhead watering to drip irrigation or sub-irrigation is likely to greatly reduce disease incidence.

- Some strains of *P. clematidina* are resistant to benzimidazole fungicides. Chemical control can potentially be improved by the use of more effective fungicides such as difenoconazole and strobilurin fungicides. Note : Use of these chemicals is currently not permitted on protected clematis; they may be used only on outdoor crops at growers own risk under the Long Term Arrangements for Extension of Use. Varieties may differ in their reaction to fungicide treatment.

©1999 Horticultural Development Council

REFERENCES

Anonymous (1898) Clematises dying. *The Garden*, 54(1394), 99.

Arthur, J.C. (1885) Disease of Clematis. In *New York State Agricultural Experiment Station Third Annual Report (1884)*. Geneva, N.Y.: New York State Agricultural Experiment Station. pp. 383-385.

Bemann, W. (1968) *Studien über die Pathologie des Clematis-Sterbens*. Dissertation, Friedrich Schiller Universität, Jena, Germany.

Bird, P.M. & Ride, J.P. (1981) The resistance of wheat to *Septoria nodorum*: fungal development in relation to host lignification. *Physiological Plant Pathology*, 19, 289-299.

Blok, I. (1965) Verwelkingsziekte in Clematis. In *Jaarboek 1964 Proefstation voor de Boomkwekerij*. Boskoop: Proefstation voor de Boomkwekerij. pp. 82-87.

Boerema, G.H. (1993) Contributions towards a monograph of *Phoma* (Coelomycetes) II. Section *Peyronellaea*. *Persoonia*, 15(2), 197-221.

Boerema, G.H., De Gruyter, J. & Noordeloos, M.E. (1997) Contributions towards a monograph of *Phoma* (Coelomycetes). IV. Section *Heterospora*: Taxa with large sized conidial dimorphs, *in vivo* sometimes as *Stagonosporopsis* synanamorphs. *Persoonia*, 16(3), 335-371.

Clarkson, J.M. (1992) Fungi. In Gurr, S.J., McPherson, M.J. & Bowles, D.J. (Eds.), *Molecular Plant Pathology. A practical approach. Volume I*. Oxford: IRL Press. pp. 67-77.

Clulow, S.A., Lewis, B.G., Parker, M.L. & Matthews, P. (1991) Infection of pea epicotyls by *Mycosphaerella pinodes*. *Mycological Research*, 95, 817-820.

Comstock, J.H. (1890) The Clematis disease. *Garden and Forest*, 3, 59.

Darby, P., Lewis, B.G. & Matthews, P. (1986) Diversity of virulence within *Ascochyta pisi* and resistance in the genus *Pisum*. *Plant Pathology*, 35, 214-223.

Ebben, M.H. & Last, F.T. (1966) Clematis wilt. In *Report Glasshouse Crops Research Institute 1965*. Littlehampton: Glasshouse Crops Research Institute. pp. 128-131.

Foussat, J. (1896) Une maladie des clématites à grandes fleurs. *Le Jardin*, 10, 124-125.

Gloyer, W.O. (1915) *Ascochyta clematidina*, the cause of stem-rot and leaf-spot of Clematis. *Journal of Agricultural Research*, 4(4), 331-342.

Hafiz, A. (1951) Cultural studies on *Ascochyta rabiei* with special reference to zonation. *Transactions of the British Mycological Society*, 34(3), 259-269.

©1999 Horticultural Development Council

Howells, J. (1993) Clematis wilt. A review of the literature. *The Plantsman*, 15(3), 148-160.

Howells, J. (1994a) The genetic background of wilting Clematis. *The Clematis*, 1994, 62-67.

Howells, J. (1994b) Vulnerability to Clematis wilt in large flowered Clematis. *The Clematis*, 1994, 51-60.

Jackman, A.G. (1900) Hybrid Clematis. *Journal of the Royal Horticultural Society*, 24, 315-322.

Jones, O.W. (1981) *Clematis wilt*. Malvern: MAFF. ADAS report nr. PP/T/623.

Köller, W., Allan, C.R. & Kolattukudy, P.E. (1982) Inhibition of cutinase and prevention of fungal penetration into plants by benomyl - a possible protective mode of action. *Pesticide Biochemistry and Physiology*, 18, 15-25.

Macko, V., Staples, R.C., Yaniv, Z. & Granados, R.R. (1976) Self-inhibitors of fungal spore germination. In Weber, D.J. & Hess, W.M. (Eds.), *The fungal spore. Form and function*. New York: John Wiley & Sons. pp. 73-100.

Monte, E. & García-Acha, I. (1988) Germination of conidia in *Phoma betae*. *Transactions of the British Mycological Society*, 91(1), 133-139.

Monte, E., Martin, P.M. & García-Acha, I. (1989) Pycnidial development in *Phoma betae*. *Mycological Research*, 92(3), 369-372.

Musa, T.M. (1981) *Host-pathogen interactions between Brassica napus L. spp. oleifera (Metzger) Sink. and Leptosphaeria maculans (Desm.) Ces. et de Not.* PhD dissertation, University of East Anglia, Norwich, UK.

Renfro, B.L. & Wilcoxson, R.D. (1963) Spring black stem of alfalfa in relation to temperature, moisture, wounding, and nutrients and some observations on pathogen dissemination. *Phytopathology*, 53, 1340-1345.

Smith, G.R. (1987) *Leaf spot and wilt of Clematis caused by Phoma clematidina (Thum.) Boerema*. PhD dissertation, University of Canterbury, Christchurch, New Zealand.

Smith, G.R. & Cole, A.L.J. (1991) *Phoma clematidina*, causal agent of leafspot and wilt of *Clematis* in New Zealand. *Australasian Plant Pathology*, 20(2), 67-72.

Smith, G.R., Munro, M.H.G., Fineran, B.A. & Cole, A.L.J. (1994) Evidence for the involvement of ascochitine in phoma leafspot-wilt disease of *Clematis*. *Physiological and Molecular Plant Pathology*, 45, 333-348.

Sorauer, P. (1897) Zur Kenntnis der Clematis-Krankheiten. *Zeitschrift für Pflanzenkrankheiten*, 7, 255-256.

©1999 Horticultural Development Council

Spiers, A.G. (1991) *Field survey of Clematis populations in the USA, England and Europe for potential biocontrol agents to control Clematis vitalba (old man's beard) in New Zealand.* Internal report, DSIR, Palmerston North, New Zealand.

Spiers, A.G. (1994) *Host specificity studies of Colletotrichum gloeosporioides and Phoma clematidina, potential biocontrol pathogens of Clematis vitalba in New Zealand.* Internal report, HortResearch, Palmerston North, New Zealand.

Van de Graaf, P. (1997) Non-fungal organisms as a cause of wilt in clematis. *The Clematis*, 1997, 47-52.

Van Kuik, A.J. (1999) Val in Clematis beter beheersbaar. *De Boomkwekerij*, 16, 18-19.

Van Kuik, A.J. & Brachter, E. (1997) Schimmelziekte bij Clematis steekt opnieuw de kop op. *De Boomkwekerij*, 9, 16-17.

Vanniasingham, V.M. & Gilligan, C.A. (1988) Effects of biotic and abiotic factors on germination of pycnidiospores of *Leptosphaeria maculans* in vitro. *Transactions of the British Mycological Society*, 90(3), 415-420.

Whitehead, R. (Ed.) (1999) *The UK Pesticide Guide 1999.* Wallingford/Farnham: CAB International/BCPC.

Wolff, G.W. (1996) *An investigation into Clematis wilt.* BSc dissertation, University of Hertfordshire, UK.

Wynn, W.K. (1981) Tropic and taxic responses of pathogens to plants. *Annual Review of Phytopathology*, 19, 237-255.

Zucker, W.V. & Catara, A. (1986) Observations in the Scanning Electron Microscope on the foliar penetration of *Phoma tracheiphila*. In Cavalloro, R. & Di Martino, E. (Eds.), *Integrated pest control in Citrus-groves*. Rotterdam: A.A. Balkema. pp. 271-277.

ACKNOWLEDGEMENTS

We are grateful to all clematis growers who were involved in the project, especially to Richard Pennell of Pennells Nurseries for his help and support. Thanks are also due to the British Clematis Society and its members for their help with the survey, and to everyone else who in some way contributed to the project.

©1999 Horticultural Development Council

Table 2. Percentage of germ tubes of *Phoma clematidina* forming visible infection structures, showing subcuticular growth, and inducing halo formation on the leaf- and stem surface of two clematis varieties, 48 h after inoculation, as observed with LTSEM (n = 50).

variety	plant surface	% showing infection structures	% showing subcuticular growth	% inducing halo formation
C. 'Henryi'	lower leaf	56	12[1]	0
	upper leaf	12	0	0
	stem	26	0	0
C. montana	lower leaf	44	16	12
	upper leaf	46	14	16
	stem	52	2	4

1 in stomatal guard cells only.

As Table 2 shows, fewer visible infection structures were formed on upper leaf- and stem surface of the susceptible variety than on those of the resistant species. On the lower leaf surface, there was no clear difference in percentage of spores forming infection structures between the two varieties.

After penetrating the cuticle, some germ tubes of *P. clematidina* continued to grow subcuticularly in the epidermis of *C. montana* (see Figure 2.3). This was more common on the leaf surfaces than on the stem (see Table 2). In *C.* 'Henryi', this form of growth was found only in stomatal guard cells on the lower leaf surface. On the resistant variety, haloes within the plant cuticle were regularly observed at the site of infection structure formation (see Figure 2.4). Usually, the halo was present only directly around the penetration site, but occasionally it surrounded a whole subcuticular germ tube. Halo formation was never encountered on the susceptible variety (see Table 2).

Some germ tubes appeared to enter the plant surface without forming an infection structure, sometimes with clear signs of enzymatic dissolution of the epicuticular wax and cuticle by the fungus. Others disappeared into the thick wax layer, which prevented further observation of infection of the cuticle. On both varieties, *P. clematidina* also penetrated its host via trichomes. Germ tubes growing on the leaf surface were regularly observed entering the host at the base of trichomes, either with or without the formation of infection structures. Many spores which landed on the large trichomes germinated there and appeared to infect the plant via that route.

<u>Discussion</u>

The average number of germ tubes per spore was higher on the surface of the susceptible variety as the SEM studies showed. This could be explained by differences in nutrient availability on the leaf surface. Nutrients might increase the growth of the first germ tube formed by the spore in such a way that the development of more germ tubes is prevented.

©1999 Horticultural Development Council