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M.A. Verhaar

# Studies on biological control of powdery mildew in cucumber (*Sphaerotheca fuliginea*) and rose (*S. pannosa*) by means of mycoparasites

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BIBLIOTHEEK
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# Studies on biological control of powdery mildew in cucumber (*Sphaerotheca fuliginea*) and rose (*S. pannosa*) by means of mycoparasites

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# Stellingen

1. Zowel relatieve luchtvochtigheid als het tijdstip van toediening van *Verticillium lecanii* hebben grote invloed op het bestrijdingseffect van *V. lecanii* op echte meeldauw op komkommer.
   Dit proefschrift

2. Pindaolie lijkt een goed formuleringsmiddel voor sporensuspensies van *Verticillium lecanii* wanneer deze wordt ingezet voor de biologische bestrijding van echte meeldauw. Pindaolie verhoogt de effectiviteit van *V. lecanii*, gedeeltelijk door een verminderde afhankelijkheid van hoge relatieve luchtvochtigheid.
   Dit proefschrift

3. Studies over biologische bestrijding van komkommer- en rozemeeldauw in tritrofe systemen zijn complex. Een waterdichte controle bestaat niet. Door het toedienen van water met of zonder formuleringsstoffen wordt het fyllosfeer-systeem sterk beïnvloed.
   Dit proefschrift

4. Biologische bestrijding van komkommermeeldauw in combinatie met het gebruik van partieel resistente komkommerrassen heeft meer kans van slagen dan beide methoden afzonderlijk.
   Dit proefschrift

5. Onder natuurlijke omstandigheden is biologische ziektebeheersing zelden spectaculair en zij blijft vaak onopgemerkt. Deze biologische ziektebeheersing wordt pas opgemerkt wanneer het evenwicht in het systeem wordt verstoord, bijvoorbeeld na eliminatie van antagonisten door pesticiden.
   Baker K.F. and Cook R.J., 1974. Biological control of plant pathogens. W.H. Freeman and Company, San Francisco, USA; Hofman T.W., 1988. Effect of granular nematicides on the infection of potatoes by *Rhizoctonia solani*, Wageningen , Academisch proefschrift; Raaijmakers J.M., 1998. Natural plant protection by 2,4-diacetylphoroglucinol producing *Pseudomonas* spp. in take-all decline soils. Molecular Plant-Microbe Interactions 11: 144-152.

6. Het experimentele resultaat dat, tegen de verwachting in, bij hoge relatieve luchtvochtigheid infecties van komkommerplanten door *Botrytis cinerea* minder ernstig zijn dan bij lage relatieve luchtvochtigheid biedt perspectieven voor biologische bestrijding van echte meeldauw op komkommer.
   Dik A., 1998. Geïntegreerde bestrijding van bovengrondse schimmels. In: Gewas-beschermingsonderzoek op de proefstations. Kerssies A. (ed.) Gewasbescherming 29: 89-90.

7. De schattingen door Oerke *et al.* van verliezen in gewasopbrengsten veroorzaakt door ziekten, plagen en onkruiden zullen in veel gevallen te hoog zijn omdat deze gebaseerd zijn op proeven waarin het desbetreffende pathogeen, plaaginsekt of onkruid wel of niet bestreden werd terwijl alle andere ziekten, plagen en onkruiden chemisch werden bestreden. Door de chemische bestrijding van een breed scala aan organismen is de kans groot dat veel van de aanwezige antagonisten van het onderzochte organisme worden geëlimineerd.
   Oerke, E.C., 1994. Estimated crop losses due to pathogens, animals petsts and weeds. In: Crop production and crop protection, Oerke, E.C., Dehne, H.W., Schönbeck F.and A. Weber (Eds.), Elsevier, Amsterdam, pp. 72-735.

8. Door nieuwsgierigheid naar het leven van anderen gedreven kijkt de Nederlander boven de 13 jaar gemiddeld 129 minuten per dag naar de televisie waardoor hij/zij de kans loopt dat het eigen leven minder interessant wordt.
   ( http/intomart/Het Media Loket)

9. De krapte op de arbeidsmarkt van de automatisering zou men kunnen oplossen door meer deeltijdbanen te scheppen waardoor het werken in de automatisering voor mensen met kleine kinderen een interessante optie wordt.

10. Schrappen van de zogenaamde onrendabele spoorlijnen door de Nederlandse Spoorwegen doet het aantal onrendabele lijnen toenemen.

11. Ruimtegebrek in het Westland zou men voor een deel kunnen oplossen door kassen boven op gebouwen te plaatsen.

12. De energie die het kost om te promoveren in de ecologische fytopathologie is de laatste jaren omgekeerd evenredig met de maatschappelijke waardering.

13. Duurlooptrainingen zijn een goed middel tegen stress.

Stellingen behorend bij het proefschrift 'Studies on biological control of powdery mildew in cucumber (*Sphaerotheca fuliginea*) and rose (*S. pannosa*) by means of mycoparasites'

M.A. Verhaar
Wageningen, 23 november 1998

*Ik adem in en kom tot rust.*
*Ik adem uit en glimlach.*
*Thuisgekomen in het nu*
*wordt dit moment een wonder!*

(Thich Nhat Hanh)

**Author's abstract**

Powdery mildew on rose (*Sphaerotheca pannosa*) and cucumber (*Sphaerotheca fuliginea*) are two serious diseases in glasshouses. Intensive control by fungicides is needed. The research presented here deals with biocontrol of powdery mildew on cucumber and rose by means of mycoparasites. The mycoparasites *Ampelomyces quisqualis*, *Sporothrix rugulosa*, *Tilletiopsis minor* and *Verticillium lecanii* were tested on rose powdery mildew. *V. lecanii* produced the best results. For biocontrol at glasshouse scale an efficient production method of phialoconidia of *V. lecanii* was developed. In exploratory glasshouse experiments *V. lecanii* was superior to *S. rugulosa* in controlling cucumber mildew. Especially on a partially resistant cucumber cultivar biocontrol by *V. lecanii* showed prospect. To identify characteristics for the selection of *V. lecanii* isolates with high potential for biocontrol under glasshouse conditions, the effect of water limitation on 14 isolates was explored. Conidial germination, growth and sporulation were all clearly affected by humidity, but showed no correlation with mycoparasitic potential. The effect of timing the biocontrol treatment by *V. lecanii* was studied on rooted mildewed cucumber leaves. At high humidity early preventative and early curative treatments gave considerable reduction in mildewed leaf area. In semi-commercial glasshouse experiments our *V. lecanii* isolate, *A. quisqualis and Sporothrix flocculosa* were tested against cucumber powdery mildew. *A. quisqualis* did not control the disease. *V. lecanii* had only minor effects. *S. flocculosa* gave the best control. The failure of biocontrol by *V. lecanii* was attributed to low humidity in the glasshouse. Formulation of *V. lecanii* with arachid oil significantly reduced its humidity dependence. Possibilities for integrated control are combinations of partially resistant cultivars, formulation of *V. lecanii* and combination of *V. lecanii* with fungicides.

**Voorwoord**

Voor altijd zal mijn aandacht getrokken worden door een meeldauwkolonie op een plant of boom. En nog enthousiaster zal ik worden wanneer ik zie dat er zich op dat blad een veldslag afspeelt tussen de meeldauwschimmel en andere schimmels. Het onderzoek naar biologische bestrijding van komkommer- en rozenmeeldauw heeft mij zeer geboeid.

Graag wil ik Tijmen Hijwegen en professor Zadoks bedanken voor alle discussies, adviezen en het nakijken van mijn manuscripten. Tijmen, jij hebt mij geleerd om goed naar de mycoparasieten te kijken, ik kan bijna zeggen om met ze te praten, al weet ik niet zeker of ze wel luisterden. Heel veel dank voor al die jaren samenwerking!

Tijdens een van de eerste besprekingen spiegelde professor Zadoks mij een komkommerkas voor met hier en daar een eikeboompje waarop meeldauw met daarop een mycoparasiet. Vanaf deze boompjes zou de mycoparasiet zich via de lucht door de kas verspreiden en zo de meeldauw onder controle houden. In mijn fantasie zie ik in die kas ook citroen lieveheersbeestjes die graag meeldauw knabbelen en dusdoende de mycoparasieten van de ene naar de andere meeldauw kolonie slepen. Daarnaast staan andere planten die als gastheer dienen voor biologische bestrijders. Voor biologische bestrijding van insekten worden niet alleen insekten en schimmels ingezet maar ook vogels. En zo zal de monocultuur in de kassen plaats moeten maken voor gecontroleerde stukjes "natuur". Zoals U in dit boekje kunt lezen zijn we nog lang niet zo ver, maar er is een kleine bijdrage geleverd aan het onderzoek naar de mogelijkheden van biologische bestrijding van komkommer en rozenmeeldauw.

In het voorjaar van 1992 begon ik vol goede moed mijn eerste komkommerkasproeven om de effecten van twee potentiële mycoparasieten van komkommermeeldauw te testen. Met het verzorgen en waarnemen van de planten had ik in mijn tijdsplanning rekening gehouden. Echter geen haar op mijn hoofd had er aan gedacht dat er ook nog eens duizenden komkommers geoogst moesten worden. Ik wil hier al het kaspersoneel en met name Bertus van der Laan en Wim den Dunnen bedanken voor alle hulp. Tijdens de kasproeven in Naaldwijk was Wout Hoogkamer mijn compagnon. Het waren zware maar gezellige dagen daar in die bloedhete kassen. Aleid Dik wil ik bedanken voor de gastvrijheid op het Proefstation voor Bloemisterij en Glastuinbouw te Naaldwijk en de goede samenwerking.

Naast de kasproeven werden verschillende kleinere experimenten uitgevoerd met bemeeldauwde komkommerblaadjes. Theo Damen wil ik ervoor bedanken dat ik na heel wat rondzwerven eindelijk terecht kwam bij goed instelbare klimaatkasten.

Omdat de rozenmeeldauw in Wageningen niet goed wilde aanslaan zijn in samenwerking met het Proefstation voor de Bloemisterij en Glastuinbouw in Aalsmeer proeven over biologische bestrijding met behulp van mycoparasieten uitgevoerd door studente Linda Hydra. Albert Kerssies en Tineke Boskoop, bedankt ik voor de gastvrijheid en samenwerking.

Verscheidende studenten wilden tijdens hun afstudeervak een bijdrage leveren aan dit onderzoek. Wendy van Eijnatten, Linda Hydra, Els Rooze, Pascal de Koning, Gitte Schober, Jannie Schönhagen, Ellian van Strien, Karin Østergaard en Nicole van de Werf, bedankt! Zoals jullie gemerkt zullen hebben werk ik graag met meer mensen aan een onderzoek. Zonder jullie was het niet zo ver gekomen!

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Marjan Verhaar

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# Chapter 1

# General introduction

# General Introduction

## Cucumbers and roses in the Netherlands

*Cucumber.* Cucumber is thought to have originated in India, where it was cultivated for 3000 years. Cucumbers were spread to China, Near East and Europe (Zeven and Zhukovsky, 1975). In the Netherlands, the production of cucumbers under glass started about 1900. Since 1970 introductions of more productive cultivars and improvements of cultivation techniques, such as the introduction of artificial substrate, increased the production in kg per $m^2$. Nowadays glasshouse-grown cucumber belongs to the leading vegetables of Dutch horticulture. In 1995, the area of cucumber under glass was about 750 ha with a production of 488 million kg and a value of 544 million guilders (CBS, 1998). About 85% of the produce is exported, mainly to Germany.

*Roses.* The oldest fossils of roses are about 35 million years old and are found in the mountains of N-America, Europe and Asia. In Egypt remnants of roses date from about 170 AD. Rose is thought to have originated in Persia. Roses were cultivated by the Romans and Greeks and played a role in myths and legends as symbols for youth, beauty, desire and fertility (Philips and Rix, 1993). On still lives of flowers of the Dutch and Flemish painters in the 17th and 18th century the famous roses of that time can be seen. Today, rose grown under glass is the leading cutflower in the Netherlands and a successful export product. From 1970 to 1995 the area under glass increased from 422 to 900 ha. In 1995 the 900 ha of roses under glass represented about 25% of the total area of cutflowers under glass. Rose greenhouses are heated (18-20°C) and one third of the roses is grown on artificial substrate. The production per $m^2$ is still increasing. The mean numbers of rose flowers per $m^2$ in 1985, 1990 and 1995 were 184, 223 and 248, respectively (CBS, 1998).

## Powdery mildew

Powdery mildews are characterized by the appearance of spots or patches of a white to greyish, powdery growth on the outside of plant organs. The powdery mildew fungi are biotrophic parasites. They usually grow on the plant surface, obtaining nutrients from the plant by means of haustoria reaching into the epidermal cells. An attack of powdery mildew reduces the vigour of the plant and causes a loss of crop yield. The external mycelium produces conidiophores. Each conidiophore produces a chain of conidia. Conidia are mainly carried by air currents (Frinking and Scholte, 1983). Conidia exist for 50-70% of water so that they can germinate and infect without free

water on the plant surface. When environmental or nutritional conditions become unfavourable mildews may produce one or more asci inside a closed ascocarp, the cleistothecium (Agrios, 1988). Powdery mildew fungi occur in many climates. They belong to the class *Ascomycetes*, the order *Erysiphales*, the family *Erysiphaceae*, the subfamily *Erysiphoideae*. They obtain their nutrients from the host plant, reduce photosynthesis, increase respiration and transpiration, impair plant growth, and reduce yield.

*Cucumber powdery mildew.* Cucumber powdery mildew is a major disease attacking both field- and greenhouse-grown cucumber plants. The two most frequent species on cucumber are *Erysiphe cichoracearum* DC. and *Sphaerotheca fuliginea* (Schlecht.: Fr.) Pollacci. *E. cichoracearum* was considered to be the predominant mildew pathogen throughout most of the world before 1958. Today *S. fuliginea* is more commonly reported worldwide. A shift in the predominance of these two fungi may have occurred, or the causal organism may have been misidentified in the past (McGrath and Thomas, 1996).

As cleistothecia are scarce, Ballantyne (1975) searched the available literature and developed a set of consistently reproducible criteria for the identification of cucurbit powdery mildews using the conidial stage. *S. fuliginea* was identified as the powdery mildew pathogen in the Dutch greenhouses (Boerema and van Kesteren, 1964), and so far has been the only species on Dutch cucumber. *S. fuliginea* develops quickly under favourable conditions in the glasshouses. Conidia remain viable for 7-8 days. The latent period is about 5 days (McGrath and Thomas, 1996). The name *S. fuliginea* has often been used in a wide sense comprising all or almost all *Sphaerotheca* taxa with large peridial cells. Junell (1966) split the species *S. fuliginea* and restricted the latter name to the powdery mildews on *Veronica* s.lat. Junell described cucumber powdery mildew as *Sphaerotheca fusca* (Fr.) Blumer. Braun (1985) followed Junell, and described cucumber mildew as *Sphaerotheca fusca* (Fr.) Blumer (Braun, 1995). The Royal Netherlands Society of Plant Pathology chose to use *S. fusca* (Fr.:Fr.) Blumer (Anonymous, 1996a). In this thesis the name *S. fuliginea* is used because it is still the most used name for cucumber powdery mildew in scientific literature.

*Rose powdery mildew.* Rose powdery mildew was described in 1819 by Wallroth as *Sphaerotheca pannosa* (Wallr.:Fr.) (Braun, 1995). *S. pannosa* is an economically important disease of garden and glasshouse roses. In Dutch glasshouses roses are grown year-round so that the mildew can be active throughout the year.

**Chemical control**

Intensive control by fungicides is needed, since resistant cucumber cultivars are sensitive to chlorosis and poorly adapted to use in Dutch glasshouses (Groot *et al.*, 1992). Resistant roses exist but they are not commercially important and thus rarely used. Bélanger *et al.* (1998) reported, according experiments carried out at the Research Station for Floriculture and Glasshouse Vegetables in the Netherlands, the economic damage threshold for roses to be estimated at 5 to 10 infected leaflets/m$^2$ and for cucumber a yield loss of 0.02% per percentage point of infected leaf area per plant and per day.

*Chemical control of* S. fuliginea. Up to 1965 *S. fuliginea* could be controlled effectively by protective fungicides (Besemer, 1965). The development from frames to glasshouses and the intensification of the crop facilitated the occurrence of *S. fuliginea* and protective fungicides could no longer control the disease satisfactorily. A frequent application of systemic fungicides became necessary (Schepers, 1985).

*S. fuliginea* developed resistance to several fungicides. Application of dimethirimol and benzimidazole fungicides had to be discontinued after a few years because of resistance development (Bent *et al.*, 1971; Kooistra *et al.*, 1972). The use of pyrazophos was drastically reduced when *S. fuliginea* developed partial resistance to this fungicide (Dekker and Gielink, 1979). Failure of disease control by ergosterol biosynthesis inhibitors has been observed for triforine (Schepers, 1983). McGrath and Thomas (1996) recently found isolates of *S. fuliginea* resistant to benomyl and triadimefon in commercial production fields in the USA. Mixtures and alternation of fungicide compounds are two basic strategies to reduce the resistance risk but the best strategy is to regard the use of crop production chemicals as an element in integrated crop production in which non-chemical control has first priority (De Waard, 1993).

A high input of fungicides against *S. fuliginea* is common. In 1996, seven fungicides were registered for control of cucumber powdery mildew in the Netherlands, pyrazophos, bitertanol, bupirimate, fenarimol, imazalil, tolylfluanide and triforine. Chemical control has to be started immediately after the first observation of powdery mildew (Anonymous, 1996b). Growers spray several times per season mostly at intervals of 7 to 10 days (Lohuis, 1996).

*Chemical control of* S. pannosa. Chemical control begins immediately after the first observation of powdery mildew. In 1997 benomyl, bitertanol, bupirimate, carbendazim, carbendazim/pyrazophos, dodemorf, fenarimol, imazalil, pyrazophos, thiophanate-methyl, totylfluanide, triadimenol, triflumizole, triforine and sulphur were registered for use in roses in the Netherlands. Dodemorf, fenarimol, imazalil and

sulphur were used as room treatments (Anonymous, 1996b). If necessary sprayments are repeated in intervals of 7 to 10 days (Lohuis, 1996).

Since about 1980 there is a tendency to grow greenhouse vegetables in an environmentally friendly way. With biocontrol of insects good results have been obtained. Commercial application of biocontrol on insects increased considerably. At the moment several biological agents, mostly insects, are available against whitefly, leaf miners, spider mites, thrips and caterpillars. In 98% of the cucumber area one or more insects were biologically controlled in 1996 (Anonymous, 1996c). Because fungicides can affect the equilibrium between prey and predators in biocontrol systems, fungicides against cucumber powdery mildew have to be chosen with consideration (Anonymous, 1996b).

**Alternative control treatments against cucumber and rose powdery mildew**

An enormous diversity of alternative control treatments, such as water, plant extracts, oils, antitranspirants, salts, compost extracts and biological agents were tested on powdery mildews (Bélanger *et al.*, 1998). A summary of alternative control treatments tested on cucumber and rose powdery mildew is given below.

*Water.* Yarwood (1939) suggested a control procedure with an abundant water supply for the home gardener and small commercial grower. He found that high humidity favoured growth of certain fungal parasites of powdery mildew and that rainfall, through mechanical action, materially decreased disease incidence. Water sprays have been used, with moderate success only, as a means of controlling powdery mildew, especially in rose (Perera and Wheeler, 1975; Wheeler, 1978).

Water showed to have different effects on the various stages of disease development: dryness favoured colonization, sporulation and dispersal, while high humidity favoured infection and conidial survival (Reuveni and Rotem, 1974). Yarwood (1978) found that periods of leaf wetness progressively increased mildew severity.

*Plant extracts.* Milsana®, a plant extract of *Reynoutria sachalinensis* F. Schmidt, has a disease-reducing effect on powdery mildew fungi in different crops (Herger *et al.*, 1988). This extract, marketed in Germany in 1991 (Milsana, Compo Ltd., Münster, Germany), is applied prophylactically with 7 to 10 days intervals. A weekly application of Milsana reduced disease severity of cucumber powdery mildew in eight weeks to 50% of that of the untreated control (Daayf *et al.*, 1995). Under Dutch greenhouse conditions, Milsana® clearly caused a reduction in susceptibility of cucumber plants to powdery mildew (Dik and van der Staay, 1995). The extract of

neem kernel was effective against *S. fuliginea* on courgette (Rovesti *et al.*, 1992) and garlic extract against *S. fuliginea* on cucumber (Qvarnström, 1992).

*Compost extracts.* Applications of aqueous extracts of compost were effective against various powdery mildews under which mildew on cucumber (Weltzien, 1989). The mechanism of control is believed to be induced resistance (Samerski and Weltzien, 1988). Variability in quality hampered commercial application (Bélanger *et al.*, 1998).

*Silicon amendments.* Addition of soluble silicon to nutrient solutions reduced the powdery mildew infections on cucumber (Bloemhard, 1992; O'Neill, 1991; Voogt, 1990; Menzies *et al.*, 1991). Dik (1994) observed variation in effects among cultivars and cropping seasons. Although the mechanism is not fully understood silicon has become increasingly popular in the greenhouse industry. About 60% of the cucumber growers and 30% of the rose growers in Europe are using silicon additions to the nutrient solutions (Menzies and Bélanger, 1996). Foliar sprays with potassium silicate reduced powdery mildew on cucumber (Menzies *et al.*, 1992).

*Salt applications.* Reuveni *et al.* (1996) found that spray applications with phosphate or potassium salts controlled powdery mildew in cucumber. This effect could not be replicated by Dik (Bélanger *et al.*, 1998). Spraying with sodium bicarbonate showed an inhibitory effect on *S. fuliginea* (Homma *et al.*, 1981; Ziv and Zitter, 1992) and *S. pannosa* (Horst *et al.*, 1992). The latter observed some phytotoxicity of bicarbonate on roses.

*Detergents.* Detergents such as Tween 80 and Zohar LQ-215 showed inhibitory effects on cucumber powdery mildew (Cohen *et al.*, 1996).

*Oils.* Several researchers showed an effect of various oils against powdery mildews, among which *S. fuliginea* (Härbele and Schlösser, 1993; Horst *et al.*, 1992; Ohtsuka and Nakazawa, 1991; Schneider and Northover, 1991) and *S. pannosa* (Pasini *et al.*, 1997).

*Clay and whitewash.* Spraying with a 10% suspension of clay and whitewash gave reduction in powdery mildew infections (Marco *et al.*, 1994).

*Induced resistance.* Systemic induced resistance, induced by prior inoculation of lower leaves with pathogens that cause limited local lesions, was extensively studied in cucurbits by Kuc and co-workers (Dean and Kuć, 1986). A single spray of phosphate or potassium salts on the first true leaf of cucumber was reported to induce systemic protection against powdery mildew on leaves 2-5 (Mucharromah and Kuć, 1991; Reuveni *et al.*, 1993; Reuveni *et al.*, 1995). Single applications of these salts

Table 1. Biocontrol agents tested on cucumber and rose powdery mildews

| Biocontrol agents | Mildew | References (in chronological order) |
|---|---|---|
| *Acremonium alternatum* | *S. fuliginea* | Malathrakis, 1985; Malathrakis and Klironomou, 1992; Malathrakis *et al.*, 1995 |
| *Ampelomyces quisqualis* | *S. fuliginea* | Jarvis and Slingsby, 1977; Philipp and Crüger, 1979; Sundheim, 1982; Sundheim and Krekling, 1982; Philipp *et al.*, 1984; Sztejnberg *et al.*, 1989 Schmitz-Elsherif, 1990; Feldman *et al.*, 1993 Falk *et al.*, 1995; Abo-Foul *et al.*, 1996 Dik *et al.*, 1998 [a] |
| *Aphanocladium album* | *S. fuliginea* | Hijwegen, 1988 |
| *Calcarisporium arbuscula* | *S. fuliginea* | Hijwegen, 1988 |
| *Penicillium chrysogenum* | *S. fuliginea* | Hijwegen, 1988 |
| *Sporothrix flocculosa* | *S. fuliginea* | Jarvis *et al.*, 1989; Bélanger *et al.*, 1994 Dik *et al.*, 1998 [a] |
| | *S. pannosa* | Hajlaoui and Bélanger, 1991; Hajlaoui *et al.*, 1992 |
| *Sporothrix rugulosa* | *S. fuliginea* | Jarvis *et al.*, 1989; Verhaar *et al.*, 1996 [a] |
| | *S. pannosa* | Hajlaoui and Bélanger, 1991 |
| *Tilletiopsis* spp. | *S. fuliginea* | Hoch and Provvidenti, 1979; Urquhart *et al.* 1994. |
| *Tilletiopsis albescens* | *S. fuliginea* | Knudsen and Skou, 1993; Hijwegen, 1988. |
| *Tilletiopsis minor* | *S. fuliginea* | Hijwegen, 1986, 1988, 1992 |
| *Tilletiopsis pallescens* | *S. fuliginea* | Urquhart *et al.*, 1994 |
| *Tilletiopsis washingtonensis* | *S. fuliginea* | Urquhart *et al.*, 1994 |
| | *S. pannosa* | Hajlaoui and Bélanger, 1991 |
| *Verticillium lecanii* | *S. fuliginea* | Spencer and Ebben, 1983; Hijwegen, 1988; Verhaar *et al.*, 1996 [a]; Askary *et al.*, 1997 Verhaar *et al.*, 1997 [a]; Askary *et al.*, 1998; Dik *et al.*, 1998 [a] |

[a] Included in this thesis.

also suppressed powdery mildew on roses (Reuveni *et al.* 1994). Conspicuous differences in inducibility of resistance to powdery mildew between susceptible and partially resistant cucumber and rose cultivars were found in respectively, number of colonies and number of produced spores per $cm^2$ when the synthetic inducer 2,6-dichloroisonicotinic acid (INA) was used (Hijwegen and Verhaar, 1994; Hijwegen *et al.*, 1996).

*Biological control by means of biocontrol agents.* An alternative to the use of fungicides may be biological control by means of biocontrol agents. Biocontrol can be based on competition for nutrients and space, production of antibiotics, mycoparasitism or induced resistance. Elad *et al.* (1996) reviewed biological control of powdery mildews in greenhouse crops. Table 1 provides an overview of research on potential biological control agents of *S. fuliginea* and *S. pannosa* in glasshouses during the last twenty years.

**This thesis**

The research project reported in this thesis was part of the Additional Research Programma of the Multi-Year Crop Protection Plan. The Multi-Year Crop Protection Plan published by the Dutch government aimed at a reduction of the use of fungicides by 65% in floriculture and by 50% in horticulture by the year 2010 (Anonymous, 1991 a,b,c). The control of powdery mildews on cucumber and rose, two important products of Dutch horticulture, depends mostly on fungicides. Both the development of resistance against fungicides in *S. fuliginea* and the increasing use of biocontrol agents against insects, which can be disturbed by fungicides, demand for an alternative.

The aim of the study presented here was to investigate biocontrol of powdery mildew on cucumber (*S. fuliginea*) and rose (*S. pannosa*) by mycoparasites as an alternative to chemical control. Mycoparasites are fungi growing upon other fungi, thus decreasing the growth and multiplication of the hosting fungi.

In preliminary experiments, isolates of potential mycoparasites (*Ampelomyces quisqualis, Aphanocladium album, Calcarisporium arbuscula, Penicillium* spp., *Sepedonium chrysospermum, Sporothrix rugulosa, Verticillium lecanii*) were tested on cucumber powdery mildew. In our experiments isolates of *Verticillium lecanii* and *Sporothrix rugulosa* showed the best results (Verhaar, unpublished data).

*V. lecanii* is known as a parasite of insects, nematodes and rust fungi (Schuler *et al.*, 1991). As *V. lecanii* is already used in the preparations Mycotal® against aphids and whiteflies in greenhouses in the Netherlands, a study of *V.lecanii* as a biocontrol

agent against powdery mildews in greenhouse crops could possibly lead to multifunctionality of biocontrol sprays. Therefore this study concentrated on the tritrophic system cucumber, powdery mildew and *V. lecanii*.

**Outline of this thesis**

The present thesis addresses several aspects of biocontrol by mycoparasites, gradually focusing on *Verticillium lecanii*. Chapter 2 deals with biocontrol of mycoparasites on rose powdery mildew introducing a new bioassay. The effect of relative humidity on mycoparasitism after treatments with and without various mycoparasites (*Ampelomyces quisqualis, Aphanocladium album, Sporothrix rugulosa*[1], *Tilletiopsis minor* and *Verticillium lecanii*) was studied. Chapter 3 describes an efficient method to produce high densities of phialoconidia of *V. lecanii* for biocontrol of cucumber powdery mildew. Chapter 4 discusses two exploratory glasshouse experiments on biocontrol of cucumber powdery mildew. Two mycoparasites, *S. rugulosa* and *V. lecanii*, were compared on a susceptible and a partially resistant cucumber cultivar. Chapter 5 deals with components analysis of mycopathogenicity in an attempt to select *V. lecanii* isolates with a high biocontrol capacity on cucumber powdery mildew. Chapter 6 describes experiments on preventive and curative application of *V. lecanii* for biocontrol of cucumber powdery mildew. In glasshouse experiments on a semi-commercial scale, executed at the Research Station for Floriculture and Glasshouse Vegetables in Naaldwijk, the Netherlands, three biocontrol agents (*A. quisqualis, Sporothrix flocculosa*[1] and *V. lecanii*) on cucumber powdery mildew were compared, Chapter 7. An appendix to this chapter deals with the persistence of *V. lecanii* in the glasshouse during these experiments. Chapter 8 explores the effects of formulations on humidity requirements of *V. lecanii* spores for biocontrol of *Sphaerotheca fuliginea*. Chapter 9 briefly discusses the sensitivity of *V. lecanii* to fungicides used against *S. fuliginea*. The results are integrated and discussed in Chapter 10.

[1)] In 1995 the names of *Sporothrix rugulosa* and *S. flocculosa* were changed to *Pseudozyma rugulosa* (Traquair, L.A. Shaw & Jarvis) Boekhout & Traquair and *P. flocculosa* (Traquair, L.A. Shaw & Jarvis) Boekhout & Traquair (Boekhout, 1995). In this thesis the names *S. rugulosa* and *S. flocculosa* are used for the purpose of continuity.

# Chapter 2

# Effect of relative humidity on mycoparasitism of rose powdery mildew with and without treatments with mycoparasites

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## Effect of relative humidity on mycoparasitism of rose powdery mildew with and without treatments with mycoparasites

### Abstract

A bioassay was developed to study the effectiveness of mycoparasites to control rose powdery mildew under selected environmental conditions. One isolate of *Ampelomyces quisqualis*, *Aphanocladium album*, *Sporothrix rugulosa* and *Tilletiopsis minor*, and four isolates of *Verticillium lecanii* were tested at four relative humidities (RH), 100, 90, 80 and 70%. Each of these fungi seemed to have its own, humidity-dependent pattern of mycoparasitism. Most mycoparasites lost their effectiveness rapidly below 100% RH, but one isolate of *V. lecanii* achieved over 80% mildew control at 90% RH, a hopeful result.

### Introduction

Powdery mildew, caused by *Sphaerotheca pannosa* (Wallr.:Fr.) Lév. var. *rosae* Wor., is an economically important disease of glasshouse roses. Regular fungicide applications are necessary to control the disease. Biocontrol by means of mycoparasites may be an alternative to fungicides. Several biocontrol agents have been described as candidates for the control of powdery mildews, especially of cucumber powdery mildew, *Sphaerotheca fuliginea* (Schlecht.: Fr.) Poll, such as *Ampelomyces quisqualis* Ces. (Jarvis and Slingsby, 1977; Philipp and Hellstern, 1986; Sundheim, 1982), *Aphanocladium album* (Preuss) W. Gams (Hijwegen and Buchenauer, 1984), *Sporothrix flocculosa* Traquair, L.A. Shaw & Jarvis and *Sporothrix rugulosa* Traquair, L.A. Shaw & Jarvis (Jarvis *et al.*, 1989; Verhaar *et al.*, 1996), *Tilletiopsis minor* Nyland (Hoch and Provvidenti, 1979; Hijwegen, 1986, 1992; Urquhart *et al.*, 1994) and *Verticillium lecanii* (Zimm.) Viégas (Verhaar *et al.*, 1996; Askary *et al.*, 1997). Hajlaoui and Bélanger (1991) tested the effects of temperature and humidity on the activity of *S. flocculosa, S. rugulosa* and *Tilletiopsis washingtonensis* Nyland on rose powdery mildew. *S. flocculosa,* the fastest colonizer of these three, proved to be as effective as registered fungicides under commercial conditions (Bélanger et al., 1994). Many of the known mycoparasites of powdery mildew have not yet been tested against rose mildew. Because relative humidity (RH) has been shown to exert great influence on the

effectiveness of mycoparasites (Hajlaoui and Bélanger, 1991; Jarvis *et al.*, 1989; Philipp and Hellstern, 1986), they should be tested at different relative humidities.

The objective of this study was to develop a bioassay to compare mycoparasites for effectiveness against rose powdery mildew under various environmental conditions, and to determine the effect of RH on mycoparasitism of rose powdery mildew with and without mycoparasite treatments. The potential of mycoparasites present in the resident phyllosphere mycoflora and of five mycoparasites previously reported to have a deleterious effect on cucumber mildew, *A. quisqualis*, *A. album*, *S. rugulosa*, *T. minor* (one isolate each), and *V. lecanii* (four isolates) were tested at four relative humidities, ranging from 100% to 70%.

## Materials and methods

*Bioassay*

*Cut rose leaves*. Compound rose leaves (cv. Sonia) with just unfolded leaflets were taken from plants grown in a glasshouse, in which no fungicides against powdery mildew had been used for two weeks prior to the experiment. Only the top three leaflets were used, the lower leaflets were removed. A test unit was prepared by sticking the petiole of a leaf through a small hole in the cap of a plastic test-tube containing about 5 ml of a sucrose solution. Two ranges of sucrose solutions (0; 0.001; 0.005; 0.01; 0.015; 0.02% and 0; 0.1; 0.5; 1; 1.5; 2%) were tested to determine the optimum for shelf life of the mounted leaves. Per litre of sucrose solution, 0.5 mg $AgNO_3$ was added to prevent the growth of algae. The test units were incubated in a climate room (20°C; 16 hours light, 20 $W/m^2$, Philips Son-T Agro 400 W; 80% RH). Twice a week the nutrient solutions were re-adjusted to their original volumes. Per sucrose solution, five test units were assessed and the experiment was conducted three times.

*Inoculum effectiveness*. Before mounting, detached rose leaves were inoculated in a vacuum-operated settling tower (Reifschneider and Boiteux, 1988) at a pressure of -15 kPa by laying them, with the upper surfaces upwards, at the bottom of the tower. Pieces of leaves with different numbers (1, 5 or 10) of abundantly sporulating mildew colonies (diameter 5-10 mm) were used as inoculum sources to determine the optimum inoculum density. After a sedimentation time of 2 min the inoculated leaves were removed from the tower and mounted. Inoculations were performed in order from low to high levels of inoculum. Per

mildew colony, the spore deposit was about 7 spores per $cm^2$. Test units containing 0.01% sucrose solution were incubated for 3 days in plastic boxes (43 x 35 cm, 23 cm high), with a shallow layer of water on the bottom. The boxes were covered by polyethylene sheets to obtain a saturated atmosphere, optimal for germination of *S. pannosa* (Wheeler, 1978). The boxes were placed in a growth chamber (20°C; 16 hours light, 20 W/$m^2$, Philips Son-T Agro 400 W). After 7 days the test units were taken from the boxes and incubated for 14 days in a growth chamber with a RH of 75% during the day and 85% at night. Twelve test units were inoculated per inoculum level. The number of mildew colonies was assessed per leaflet. The experiment was conducted three times.

*Mildew control by mycoparasites*

*Mycoparasites.* The mycoparasites are listed in Table 1. *V. lecanii* isolates 1, 4, 13 and 14 were cultured on oatmeal agar, *A. album* and *S. rugulosa* on malt agar, and *A. quisqualis* on oatmeal agar alternating with malt agar. *T. minor* was cultured in a liquid medium of 2% malt extract and 0.2% mycological peptone in the dark at 20 °C in a rotary shaker at 140 rpm (Hijwegen, 1986). Spore suspensions were prepared from 10 to 14 days old cultures by washing the colonies with distilled water and filtering the resulting suspension through cheesecloth. In the case of *T. minor*, an 8 days old culture was taken. Spore densities were determined with a haemocytometer and adjusted to $5\times10^6$ spores $ml^{-1}$. As an additive, 0.01% Algan-S, a biological detergent containing 30% *Ascophyllum nodosum* extract (Europlant Ltd, Appelscha, the Netherlands), was used.

*Mildew.* About 60 detached rose leaves were inoculated simultaneously as described before with *S. pannosa* using 10 colonies as inoculum. They were mounted in plastic test-tubes and incubated at a saturated atmosphere as described above.

*Biocontrol treatments.* After seven days of incubation, inoculated leaves with moderate levels (2-4 colonies per leaflet) of mildew were selected and randomly distributed over the treatments. These leaves were sprayed with spore suspensions of the mycoparasites containing $5\times10^6$ spores per ml using a DeVilbiss sprayer in a separate building. Controls were sprayed with distilled water with or without 0.01% Algan-S (Wa and W, respectively). Thereafter the leaves were incubated in climate rooms (280$m^3$, under Philips Son-T Agro 400 Watt assimilation lamps (20 W/$m^2$, 16 hours light); 20°C; 70%, 80%, 90% or 100% RH controlled by ultrasonic mist equipment Damfomat Edo20; windspeed (2 m/sec). In practice temperatures ranged between 19 and 21°C and RH's (except 100%) varied

Table 1. The mycoparasites tested against *Sphaerotheca pannosa* on rose.

| Code | Isolate | Year of isolation | Host/origin |
|---|---|---|---|
| Aa | *Aphanocladium album* | 1985 | ex *Sphaerotheca fuliginea* on *Cucumis*, Wageningen, The Netherlands |
| Aq | *Ampelomyces quisqualis* | 1993 | ex *Sphaerotheca pannosa*, Republic of South Africa |
| Sr | *Sporothrix rugulosa* | 1990 | ex *Sphaerotheca fuliginea* on *Cucumis*, Wageningen, The Netherlands |
| Tm | *Tilletiopsis minor* | 1981 | ex *Erysiphe martii* on *Lupinus polyphyllus*, Bonn, Germany |
| V1 | *Verticillium lecanii (1)* | 1988 | ex *Sphaerotheca fuliginea* on *Cucumis*, Wageningen, The Netherlands |
| V4 | *Verticillium lecanii (4)* | 1973 | CBS 470.73 ex *Hemileia vastatrix* on *Coffea*, India |
| V13 | *Verticillium lecanii (13)* | 1993 | ex *Erysiphe* on *Hordeum*, Wageningen, The Netherlands |
| V14 | *Verticillium lecanii (14)* | 1993 | ex *Microsphaera* on *Mahonia*, Marienthal, Germany |

within 5%. To obtain a saturated atmosphere in the growth chambers, polyethylene covered plastic boxes were used as described above.

Percentage parasitized powdery mildew was assessed at 3 and 7 days after biocontrol treatments (d.a.b.) for 100% RH, and 2, 6 and 14 d.a.b. for the other RH values. The percentage of the mildew colonies parasitized by the biocontrol agents was assessed using a binocular microscope at x50 magnification. Percentage parasitation was scored in classes separated by 5% intervals (0,5,10,...). The mean of the three leaflets per test unit was calculated and subjected to statistical analysis.

For each humidity level, two or three sequentially replicated experiments were carried out with a total of 20 or 21 leaves per mycoparasite. Test units were randomly placed on a table in the middle of the growth chamber.

*Additional check on cross-contamination*

After the biocontrol treatments were finished, the growth chambers were cleaned so that no cross-contamination could occur. Per humidity level, 20 rose leaves were

inoculated with mildew and the percentage parasitized mildew colonies were assessed after two weeks.

*Statistics*

The effect of the amount of mildew inoculum on the mean number of mildew colonies per leaflet was analyzed by ANOVA and mean results per inoculum per experiment were subjected to nonlinear regression analyses.

Because humidity effects on mycoparasitism were obvious, the data were separated by humidity level and subjected to the Kruskal-Wallis test with an associated multiple range test for unequal sample sizes (Hollander and Wolfe, 1973).

Percentages parasitized mildew colonies increased at rates varying according to mycoparasite, RH level, and time. The rate of increase of parasitism expressed in percentage/day was calculated by interpolation between observations made at the days (d.a.b.) indicated above.

## Results

*Bioassay, inoculum effectiveness*

Rose leaves grown on high sucrose concentrations, 1, 1.5 and 2%, yellowed and died. On a sucrose concentration of 0.01% leaves remained green over a three week period.

The number of mildew colonies used as inoculum had a significant ($P \leq 0.05$) effect on the resulting number of lesions per leaflet. The effect of number of inoculum colonies on resulting number of mildew colonies could be roughly described by the nonlinear equation: $Y = 3.08X/(0.42 + X)$ ($R^2 = 0.60$, $n = 12$, $P \leq 0.01$). The differences were relatively small, probably due to the inevitable background infection caused by spores dispersed in the glasshouse environment. The mean background infection of about 1 colony/leaflet, was evidenced by the 0 inoculum level. For further research 10 colonies were used as the inoculum dosage, resulting in about 3 colonies per leaflet.

*Mildew control at different relative humidities*

In all cases the applied mycoparasites were observed on treated mildew colonies, while no equivalent amounts of saprophytic fungi or other mycoparasites were noticed on these colonies.

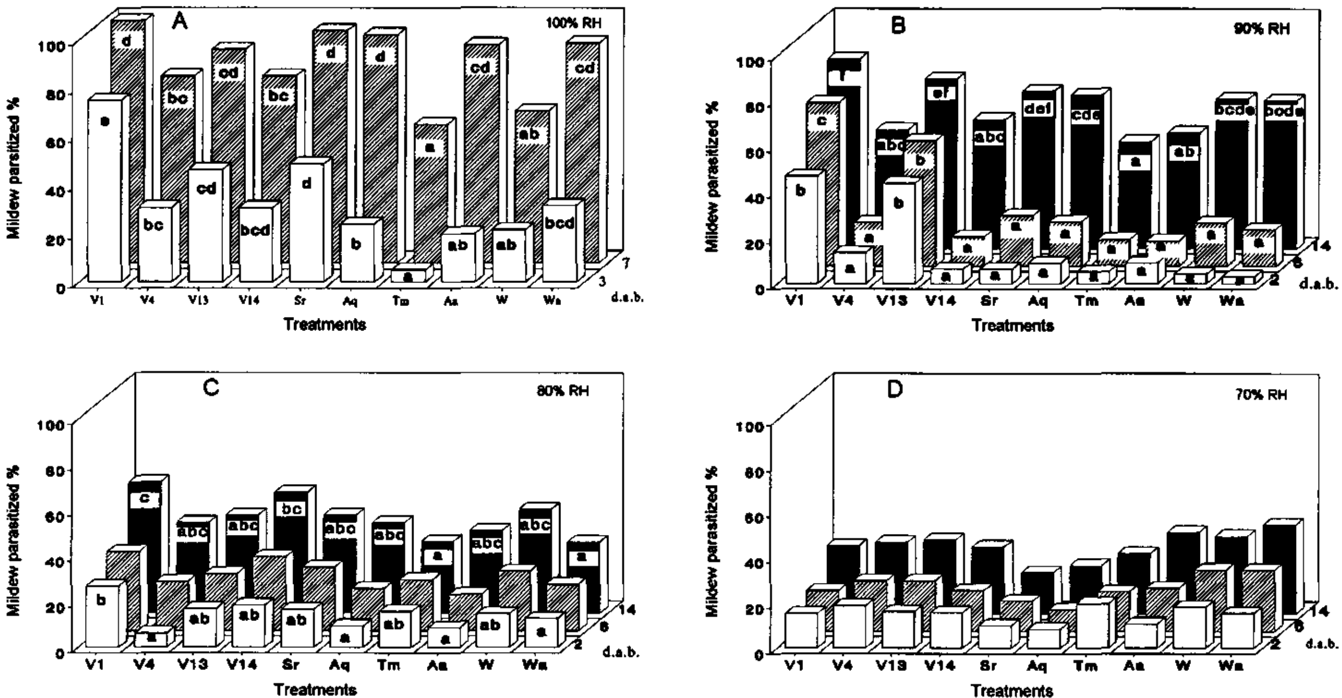


Figure 1. Mildew parasitation 3 and 7 days after application of biocontrol agents at 100% RH (A) and 2, 6 and 14 days after application at 90 (B), 80 (C) and 70% (D) RH The biocontrol agents are listed in table 1. Water (W) and water + Algan-S (Wa) served as controls. Entries are means of 2 or 3 experiments with a total of 21 units. Values per assessment date with different letters are significantly different according a multiple range test based on Kruskal-Wallis rank sums ($P \leq 0.05$).

*100% RH.* At a RH of 100%, mildew parasitation by *V. lecanii*-1 was over 70% within 3 d.a.b. (Fig.1A). The other three *V. lecanii* isolates attained similar values only at 7 d.a.b. At least ninety percent of the mildew area was parasitized by *V. lecanii*-1, *V. lecanii*-13, *S. rugulosa, A. quisqualis, A. album* and the water plus Algan-S control treatment at 7 d.a.b.

*90% RH.* At 90% RH, *V. lecanii*-1 and *V. lecanii*-13 attained values over 40% within 2 d.a.b., while at 6 d.a.b. the mean values were 72 and 55% (Fig.1B). The other mycoparasites did not reach more than 25% parasitized mildew at 6 d.a.b. At 14 d.a.b. the percentages mildew parasitized by *V. lecanii*-1 and *V. lecanii*-13 had risen to 83% and 75%, while *S. rugulosa* and *A. quisqualis* attained about 70%.

*80% RH and 70% RH.* At 80% RH *V. lecanii*-1 parasitized 26% at 2 d.a.b. and 57% at 14 d.a.b (Fig.1C). At 70% RH mildew parasitation never surpassed 35% and no significant differences between mycoparasites were found (Fig.1D).

*Expansion rates.* The expansion rates of the mycoparasites on mildew, expressed in percentage/day, differed most in the first two days, the period of establishment of the mycoparasites. At 100% RH *V.lecanii*-1, *V. lecanii*-13 and *S. rugulosa* were the fastest colonizers during the first 2 days. Other mycoparasites such as *A. album* and *A. quisqualis* started slowly and showed increasing expansion rates after 2 d.a.b. At 90% RH the expansion rates of *V. lecanii*-1 and *V. lecanii*-13 during the first 2 d.a.b. were comparable to those at 100% RH The other mycoparasites developed more slowly. At 80% RH *V. lecanii*-1 was one of the fastest mycoparasites. As the two most promising isolates of *V. lecanii* (isolate 1 and isolate 13) and the two control treatments did not differ significantly ($P \leq 0.05$), the mean expansion rates of these two *V. lecanii* isolates and the two control treatments were summarized in Table 2.

*Control treatments.* The water and water plus Algan-S control treatments resulted at 100% RH and 90% RH in high percentages parasitism at respectively 7 and 14 d.a.b. (Fig.1A, 1B). *V. lecanii*, *Penicillium* spp. and *Cladosporium* spp. were frequently observed on the mildew of the control leaves. At 100% RH *S. rugulosa* was also found.

*Additional check on cross-contamination.* At 100% RH about 30% parasitized mildew was observed 14 d.a.b. A *Penicillium* sp. was isolated from parasitized mildew. At the other humidities amounts of parasitized mildew were negligible.

**Table 2.** Expansion rates of *V. lecanii* and of the present phyllospheric mycoflora on *S. pannosa* in percentage per day over two or three observation periods, after respectively biocontrol treatments with *V. lecanii* (means of isolate 1 and 13) and control treatments (means of water and water plus 0.01% Algan-S).

| | | rate (mm/day) | |
|---|---|---|---|
| % RH | Period | *V. lecanii* | Control |
| 100 | 0-3 | 20.2 a[a] | 8.3 b |
| | 4-7 | 11.3 | 10.8 |
| 90 | 0-2 | 22.5 a | 1.8 b |
| | 3-6 | 4.7 | 3.5 |
| | 7-14 | 2.1 a | 5.8 b |
| 80 | 0-2 | 11.0 | 7.0 |
| | 3-6 | 2.5 | 2.5 |
| | 7-14 | 2.6 | 1.9 |

[a] Means within a row with different letters are significantly different according to a multiple range test based on Kruskal-Wallis rank sums ($P \leq 0.05$).

## Discussion

*Bioassay*

Beyond a certain dosage of mildew inoculum the number of resulting mildew colonies seemed to be stable (Hijwegen *et al.*, 1996). A maximum amount of about 5 colonies per leaflet was regularly observed on cv Sonia in glasshouses (Kerssies, unpublished). This phenomenon of a stable number of resulting colonies has not yet been explained.

As rose leaves become rapidly resistant to mildew, *S. pannosa* can only infect young leaves (Wheeler, 1978; Frinking and Verweij, 1989). One week after inoculation the diameter of the mildew colonies had attained 5 to 10 mm and hardly increased further. Thus, mildew colonies in all treatments were comparable in size and maturity.

The bioassay using detached rose leaves, kept on a 0.01% sucrose solution, proved to be a useful system to observe different mycoparasites of *S. pannosa* during a two week period at various humidity conditions.

The relative high infections on the mildew of the controls could have originated from (1.) cross-contamination between test units within one compartment, (2.) micro-organisms resident on the rose leaves, and (3.) cross-contamination between compartments. The first explanation is the most probable. The negative conclusion is that control units placed at random among the treated units do not serve their purpose well. The positive conclusion could be that spontaneous dispersal of biocontrol agents is highly effective, at least within the range of time (1 and 2 weeks) and distance (0.1-1m).

*Mildew control*

*General comments.* The experiments were performed in a research station for floriculture and mildew inoculum as well as leaves for the test units were taken from plants growing in a semi-commercial glasshouse. Per humidity level, all treatments including the controls were located in the same climate room. This conditions can explain the relatively high parasitism levels by *V. lecanii* and the slightly parasitic saprophytes *Cladosporium* and *Penicillium* (Hijwegen, 1992) in the water and water plus Algan-S control treatments, after more than 6 days. Micro organisms on the rose leaves as *Cladosporium* and *Penicillium,* though only weakly parasitic and slow growing, can degrade a substantial amount of mildew in the long run, due to their ability to germinate and grow at RH's of 80-85% and enormous reproduction capacity (Hijwegen, unpublished). Enhancement of the effectiveness of the resident phyllosphere mycoflora through creating favourable environmental conditions by application of a formulation without a biocontrol agent was mentioned by Yang *et al.*(1993) and Verhaar *et al.* (1996). Rogers (1959) noticed that prolonged wetting of mildewed leaves caused the collapse of conidial chains. Probably, at a RH of 100%, collapsed conidiophores provide nutrients for the resident mycoflora.

The additional check on cross-contamination showed parasitation of 30% at 14 d.a.b., but only at 100% RH, whereas the water control treatments in the main experiment showed about 60% parasitized mildew ≥7 d.a.b., at relatieve humidities ≥ 90%. This indicates that cross-contamination in the main experiment by far exceeded the effect of the resident mycoflora.

*Mycoparasites.* At 100% RH most mycoparasites parasitized a considerable area of mildew within 6 d.a.b., while most mycoparasites showed less activity at

lower humidities. *T. minor*, even at 100% RH, gave a rather poor control. A relative humidity close to saturation is needed for growth of this fungus (Urquhart *et al.*, 1994; Hajlaoui and Bélanger, 1991; Hijwegen, 1992). On *T. minor* treated leaves, where saprophytes and other mycoparasites have reduced opportunities, due to excess *T. minor* inoculum, biocontrol is to slow, at least in the early stages, that the *T. minor* treatment could be seen as a control treatment. Possibly a better control treatment than the water controls. When antagonists are antagonized the net effect can be next to nil (Verhaar *et al.*, 1996). For *A. quisqualis* and *A. album* the low activity below 100% RH is in accordance with the results of Philipp *et al.* (1990) and Hijwegen and Buchenauer (1984). *S. rugulosa* showed little activity at 90% RH Hajlaoui and Bélanger (1991), however, obtained more than 80% rose powdery mildew parasitized by *S. rugulosa* at 6 d.a.b., 90% RH and 26°C, but only 30% at 18°C, a value comparable to ours at 20°C. In our experiment, performed at 20°C, *V. lecanii*-1 and *V. lecanii*-13 seemed to be the fastest powdery mildew colonizers at 90% RH at 6 d.a.b.

The differences in expansion rates between mycoparasite treatments indicate that every mycoparasite had its own, humidity-dependent, pattern of parasitism. Considerable differences in activities were noted for the four *V. lecanii* isolates. This result underlines the importance of testing different isolates of each mycoparasite.

*Possible improvements.* In Dutch rose glasshouses the daily mean RH fluctuates between 50% in winter and 90% in fall. In spring and summer the temperature and the RH show a daily pattern, low temperature (<20°C) and high RH (>80%) at night and high temperature (>20°C) and low RH (<70%) during the day (Kerssies, 1994). These conditions are far from optimal for biological control. An emulsion or a carrier may be needed to provide moisture for the mycoparasites. Mineral oils (Philipp *et al.*, 1990) or invert emulsions (Yang *et al.*, 1993), for instance, could reduce moisture stress of *A. quisqualis* and the mycoherbicides *Alternaria alternata* and *A. angustiovoidea*. With the addition of a vegetable oil the control effect of *V. lecanii* could be greatly increased (Chapter 8).

In conclusion, the bioassay developed to study mycoparasitism on detached mildewed rose leaves at different RHs indicated that two isolates of *V. lecanii* are interesting as potential biological control agents of *S. pannosa*. Experiments in commercial rose glasshouses are necessary to determine the biocontrol potential under more realistic conditions.

# Chapter 3

## Efficient production of phialoconidia of *Verticillium lecanii* for biocontrol of cucumber powdery mildew, *Sphaerotheca fuliginea*

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**Efficient production of phialoconidia of *Verticillium lecanii* for biocontrol of cucumber powdery mildew, *Sphaerotheca fuliginea***

**Abstract**

A method is described that yields over 3 x $10^9$ phialoconidia/ml of *V. lecanii*.

Spore production of mycoparasitic fungi may vary considerably and mass production of fungal conidia for biological control often meets with problems. Some mycoparasites do not produce sufficient inoculum for small-scale experiments (Hijwegen, 1988). Usually, special studies on cultivation methods are required as demonstrated for *Ampelomyces quisqualis* by Schmitz-Elsherif (1990).

*Verticillium lecanii* (Zimm.) Viégas strain Fyto 88.1, isolated from *Sphaerotheca fuliginea* (Schlecht.: Fr.) Poll. (Hijwegen, 1988), can easily be grown and sporulates well on most solid media such as agars containing potato-dextrose broth, malt extract plus mycological peptone, oat meal, chitin or skimmed milk. In this way productions of 1-4 x $10^7$ conidia/$cm^2$ (0.5-2 x $10^9$ conidia/petridish) can be obtained. When, however, 8 x $10^{10}$ conidia were required every week for a greenhouse experiment, (Verhaar and Hijwegen, unpublished) surface grown cultures were inadequate and other methods of cultivation were investigated. *V. lecanii* was grown at 20°C and 135 rpm in liquid media containing milled, autoclaved oat meal, skimmed milk or malt extract plus mycological peptone. Yields of conidia were rather low. Conidium production was enhanced by raising the incubation temperature to 25°C. Yeast cell walls and malt extract plus mycological peptone gave a good yield of conidia. These media were, however, surpassed by far by growing *V. lecanii* in autoclaved oat meal suspension.

*V. lecanii* has been reported to produce only blastoconidia in liquid culture (Latgé et al., 1986). After consulting Dr. W. Gams, CBS, Baarn this was investigated using our strain Fyto 88.1. In our experiments submerged mycelium, producing micro-verticils with micro-phialides carrying normal phialoconidia, was formed in liquid culture. Budding was not observed. Conidia produced in liquid culture could not be distinguished microscopically in size or shape from conidia produced on solid oat meal medium. In 1% oat meal more mycelium was formed than in 3% oat meal. This raises the question whether oat meal contains substances that influence the mycelium / conidia ratio.

To investigate conidium production quantitatively, 0.8 $cm^2$ pieces of agar with

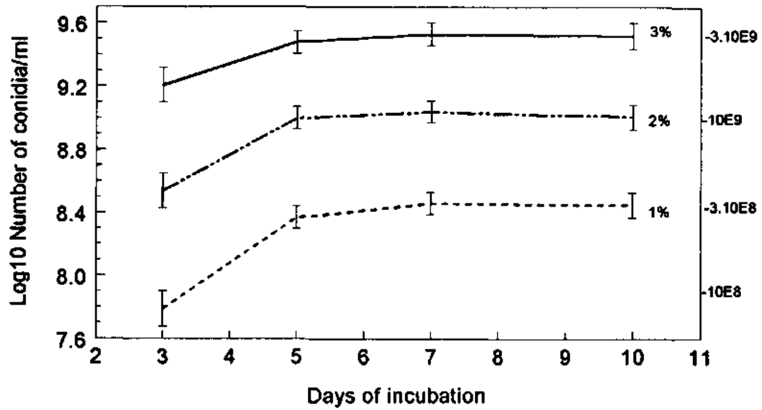


Figure 1A. Effect of oat meal concentrations (1%, 2% and 3%) on the number of *V. lecanii* conidia produced in liquid media incubated at 25 °C. The graph gives the mean number of conidia/ml in four replications. Data were log transformed (y-axis) and back-transformed (right y-axis). The standard errors of the mean are given in the graphs by bars

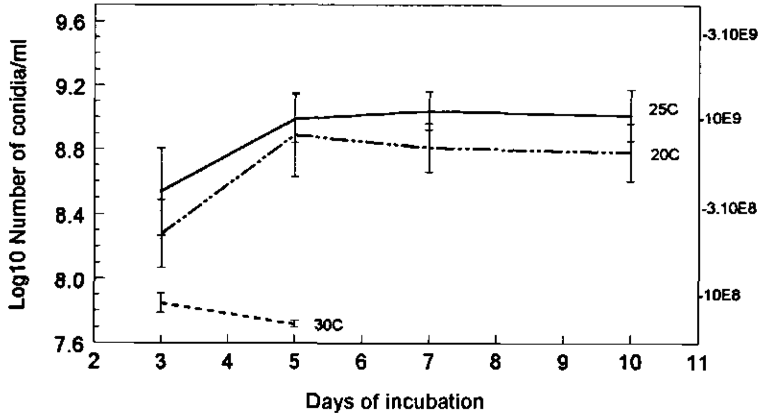


Figure 1B. Effect of incubation temperatures of 20, 25 or 30 °C on the number of *V. lecanii* conidia produced in 2% liquid oat meal medium. The graph gives the mean number of conidia/ml in four replications. Data were log transformed and back transformed (right y-axis). The standard errors of the mean are given in the graphs by bars.

mycelium from the margin of a growing culture of *V. lecanii* were added to 300-ml Erlenmeyer flasks containing 100 ml of 1, 2, or 3% milled and autoclaved oat meal (Quaker HO Naturel) in distilled water. These flasks were incubated in the dark at 25°C in a rotary shaker at 135 rpm. Other flasks containing 2% autoclaved oat meal were inoculated with *V. lecanii* and incubated in the same way at 20° or 30°C. For every treatment two flasks were used. The experiment was repeated twice. Ten-ml samples were removed under sterile conditions after 3, 5, 7 and 10 days. The suspensions were diluted 10-fold or, if necessary, 100-fold and the conidia were counted in a hemocytometer. A production of over 3 x $10^9$ conidia/ml could be obtained at 25°C in 3% oat meal (Fig. 1).

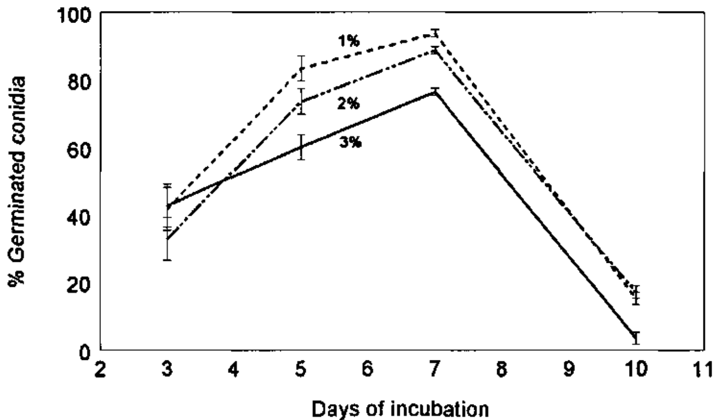


Fig. 2. Germination of conidia of *V. lecanii* produced in liquid media containing 1%, 2% or 3% oat meal, incubated at 25°C after 3, 5, 7 or 10 days incubation. Drops of 20µl containing 2 x $10^4$ spores were placed on water agar. Germination was assessed after incubation for 24 h at 20°C. The data are means of two replicates. The bars indicate the standard errors of the mean.

Germination was assessed at 3, 5, 7 and 10 days by placing 20 µl droplets containing $10^6$ conidia/ml on water agar and counting germinated and non-germinated conidia on the next day (Fig. 2).

Incubation at 30°C resulted in a limited production of conidia, which germinated poorly. Poor germination of conidia produced at temperatures above the optimum for growth does not seem to be uncommon with mycoparasitic fungi (Van Eynatten and Verhaar, unpublished). After 7 days of cultivation lysis of mycelium and conidia had occurred and the cultures had to be discarded.

As a routine procedure *V. lecanii* was cultured 7 days at 25°C and 135 rpm in a 3% oat meal suspension every week during 6 subsequent weeks. One flask per week, containing more than $10^9$ conidia/ml, was always sufficient for the 8 x $10^{10}$ conidia needed for the weekly applications on cucumber powdery mildew, *Sphaerotheca fuliginea*, during six subsequent weeks, indicating that the method is reliable.

In a later experiment 2 to 4 x $10^{10}$ conidia were required weekly. As with 3% oat meal suspensions the pores of the filter became plugged sometimes, it was decided to grow *V. lecanii* in a 1% suspension. The method proved to be reliable during the production of eight consecutive batches and 1 or 2 flasks were always sufficient for each of the weekly applications in the greenhouse experiment.

In conclusion, a high production of phialoconidia with high germinability was obtained by growing *V. lecanii* in 1 to 3% milled oat meal during 7 days at 25°C in the dark at 135 rpm.

# Chapter 4

# Glasshouse experiments on biocontrol of cucumber powdery mildew (*Sphaerotheca fuliginea*) by the mycoparasites *Verticillium lecanii* and *Sporothrix rugulosa*

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# Glasshouse experiments on biocontrol of cucumber powdery mildew (*Sphaerotheca fuliginea*) by the mycoparasites *Verticillium lecanii* and *Sporothrix rugulosa*


## Abstract

Two potential biological control agents of cucumber powdery mildew (*Sphaerotheca fuliginea)*, *Verticillium lecanii* and *Sporothrix rugulosa*, were tested under glasshouse conditions. Two experiments were carried out. In the first experiment two cucumber varieties with different levels of resistance, cv Corona (susceptible) and cv Flamingo (partially resistant) were used. *Verticillium lecanii* controlled the mildew better than *S. rugulosa*. On cv Flamingo, *V. lecanii* could keep the mildew severity below 15% infected leaf area for 9 weeks after inoculation with *S. fuliginea*. Treatment by Hora Oleo 11E, alone or as an additive to *V. lecanii*, was as good as a fungicide treatment. In the second experiment weekly and two-weekly treatments with *V.lecanii* were compared on cv Flamingo. Weekly treatments with *V. lecanii*, kept mildew severity at a level below 20% infected leaf area during 10 weeks after inoculation with *S. fuliginea*. If applied to a partially resistant cucumber cultivar, *V. lecanii* is an interesting candidate for biological control of *S. fuliginea*.


Key Words: *Sphaerotheca fuliginea, Sporothrix rugulosa, Verticillium lecanii*, biocontrol, Hora Oleo 11E, partial resistance.


## Introduction

Mycoparasites might be used in biological control of powdery mildews in glasshouses. Several mycoparasites of cucumber powdery mildew are known (Hijwegen, 1986; Hoch and Provvidenti, 1979; Jarvis *et al.*, 1989; Malathrakis, 1992; Philipp *et al.*, 1984; Sundheim, 1982; Urquhart *et al.*, 1994). The potential of mycoparasites to control powdery mildew depends on their intrinsic properties and environmental conditions. Philipp *et al.* (1984) noticed that intensive wetting during inoculation was imperative to obtain high rates of parasitation. Hajlaoui and Bélanger (1991) found that temperature and humidity influenced the colonization of rose powdery mildew by three antagonists of this fungus.

In commercial glasshouses, conditions are rarely favorable to a good development of mycoparasites. Relative humidity during daytime is low (around

75%) and temperatures can rise to high levels. Formulations could be used to improve the parasites' control potential by reducing their humidity demand (Hijwegen, 1992; Philipp *et al.*, 1990). Also, the search must be for a mycoparasite with a high control potential under glasshouse conditions and for improvement of these conditions for mycoparasitism. A partially resistant cucumber variety might slow down mildew development and thus favor mycoparasitism.

In preliminary experiments with *Ampelomyces quisqualis, Sporothrix rugulosa, Tilletiopsis minor,* and *Verticillium lecanii* the best results were obtained with *S. rugulosa* and *V. lecanii* on mildewed leaves in plastic boxes with a relative humiditiy of about 85% (Verhaar, unpublished). Most experiments on record were done with detached mildewed leaves or young, mildewed cucumber plants (Hijwegen, 1986, 1992; Hoch and Provvidenti, 1979; Jarvis *et al.*, 1989; Malathrakis, 1992; Philipp *et al.*, 1984, 1990). This paper reports experiments on biocontrol of cucumber powdery mildew under glasshouse conditions, applying *V. lecanii* (Zimm.) Viégas and *Sporothrix rugulosa* Traquair, Shaw and Jarvis, to cucumber varieties with different levels of resistance to mildew (*Sphaerotheca fuliginea* (Schlecht.: Fr) Poll.). Preliminary results were published elsewhere (Verhaar *et al.*, 1993).

## Materials and methods

### Plants

Plants of the susceptible cucumber cv Corona and the partially resistant cv Flamingo were raised in pots in growth chambers (20°C, 70% RH). After 3 weeks they were transplanted without pots into the soil of a glasshouse. Plant distance was 40 cm in the rows and 1 m between the rows. Resistant plants of cv Profito were planted along the borders to minimize border effects.

### Mildew

In Experiment 1 (Exp-1) leaves seven and eight of 6-week-old plants and in Experiment 2 (Exp-2) leaves six and seven of 7-week-old plants were inoculated with *S. fuliginea* by spraying a suspension of $10^5$ spores per ml in tap water, which resulted in about 60 spores per $cm^2$ leaf surface.

**Mycoparasites**

*Sporothrix rugulosa* was isolated in 1990 from mildewed cucumber plants and cultured on malt extract agar in petri dishes. After 1 week the spores were harvested by washing the petri dishes with tap water and filtering the spore suspensions through cheesecloth. *Verticillium lecanii* strain F88.1 was isolated from cucumber mildew in 1988 and cultured according to Verhaar and Hijwegen (1993). One-week-old cultures were diluted and filtered through cheesecloth, to separate mycelium and spores. In Exp-1, spore suspensions of the mycoparasites ($5.10^6$ spores per ml in water with 20 µl Tween 80 per liter), with or without 0.5% vv Hora Oleo 11E ("Hora", a formulated paraffin oil of Ciba Geigy), were applied weekly as biocontrol agents, whereas 0.5% vv Hora and a fungicide (fenarimol, Rubigan ®, AgrEvo, Haren, The Netherlands) were used as controls (Table 1). In Exp-2, spore suspensions of *V. lecanii* ($5.10^6$ spores per ml water with 20 µl Tween 80 per liter) were applied once or twice per fortnight. Hora (0.5% vv) and water were used as controls (Table 1). In both experiments the untreated control was omitted to avoid the 'cryptic error' (Vanderplank, 1963) due to high levels of inoculum which cause cross-contamination between plots.

In Exp-1 and Exp-2 treatments began 1 week after the first observation of mildew lesions and continued for 6 and 8 weeks, respectively. Treatments were

Table 1. The Treatments

| Experiment 1 | | Code |
|---|---|---|
| *Verticillium lecanii* ($5.10^9$ spores per liter water with 20µl Tween 80 per liter) | | V |
| *Sporothrix rugulosa* ($5.10^9$ spores per liter water with 20µl Tween 80 per liter) | | S |
| Hora Oleo 11E (5 ml per liter) | | H |
| *Verticillium lecanii* + Hora Oleo 11E | | VH |
| *Sporothrix rugulosa* + Hora Oleo 11E | | SH |
| Fenarimol (Rubigan®) (24 mg per liter) | | F |
| **Experiment 2** | | **Code** |
| *Verticillium lecanii* | once per fortnight | V1 |
| *Verticillium lecanii* | twice per fortnight | V2 |
| Hora Oleo 11E | once per fortnight | H1 |
| Hora Oleo 11E | twice per fortnight | H2 |
| Water (+20µl Tween 80 per liter) | once per fortnight | W1 |
| Water (+20µl Tween 80 per liter) | twice per fortnight | W2 |

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P  P  P  P  P  P  P  P  P  P          P  P  P  P  P  P  P  P  P
P| C1 C1 C1 F5 F5 F5 F6 F6 F6 |    | F5 F5 F5 C6 C6 C6 F2 F2 F2 |P

P| C6 C6 C6 F4 F4 F4 C4 C4 C4 |    | F1 F1 F1 C2 C2 C2 C3 C3 C3 |P
P| C2 C2 C2 C5 C5 C5 F1 F1 F1 |    | C5 C5 C5 F6 F6 F6 F4 F4 F4 |P

P| C3 C3 C3 F2 F2 F2 F3 F3 F3 |    | F3 F3 F3 C4 C4 C4 C1 C1 C1 |P
P| F1 F1 F1 F6 F6 F6 C2 C2 C2 |    | F5 F5 F5 F4 F4 F4 C4 C4 C4 |P

P| C5 C5 C5 F4 F4 F4 C6 C6 C6 |    | F6 F6 F6 C5 C5 C5 C6 C6 C6 |P
P| F3 F3 F3 F2 F2 F2 C4 C4 C4 |    | F3 F3 F3 F1 F1 F1 F2 F2 F2 |P

P| F5 F5 F5 C3 C3 C3 C1 C1 C1 |    | C1 C1 C1 C3 C3 C3 C2 C2 C2 |P
P| F3 F3 F3 F1 F1 F1 C4 C4 C4 |    | C5 C5 C5 F6 F6 F6 F5 F5 F5 |P

P| C5 C5 C5 C6 C6 C6 F2 F2 F2 |    | C1 C1 C1 C6 C6 C6 F2 F2 F2 |P
P| F6 F6 F6 C3 C3 C3 C2 C2 C2 |    | F1 F1 F1 F4 F4 F4 C3 C3 C3 |P

P| C1 C1 C1 F4 F4 F4 F5 F5 F5 |    | C4 C4 C4 C2 C2 C2 F3 F3 F3 |P
P  P  P  P  P  P  P  P  P  P          P  P  P  P  P  P  P  P  P  P
                                                                    20 m

                                                              5m
```

Figure 1. The design of Experiment 1. Letters represent cucumber cultivars: C = Corona (susceptible), F = Flamingo (partially resistant), and P = Profito (resistant). Numbers represent treatments; 1 = V+H, 2 = V, 3 = S+H, 4 = S, 5 = H, and 6 = F (V = *Verticillium lecanii*, S = *Sporothrix rugulosa*, H = Hora Oleo 11E, and F = Rubigan®). The lines represent the block borders. Experiment 2 had a similar design.

carried out with a 5 litre backpack sprayer. Per plant 125 ml liquid was sprayed till drip-off, which resulted in about 50 spores per $mm^2$ leaf area. To limit crosscontamination between plots during the spray operation, the plots in Exp-1 were separated by plastic curtains (during the spray operation only) and in Exp-2 by plants of cv Profito.

## Experimental design

Exp-1 and Exp-2 had a randomized complete block design (Gomez and Gomez, 1984) with six and four replicates, respectively. Three plants in a row represented one plot (Fig. 1).

**Mildew observations**

Percentage of leaf area covered by mildew was assessed weekly on two leaf levels per plant, using a key with ten disease classes (0 = 0% mildew, 1 = < 1%, 2 = 1-5%, 3 = 6-10%, 4 = 11-20%, 5 = 21-30%, 6 = 31-40%, 7 = 41-60%, 8 = 61-80%, 9 = 81-100%) (Ubels and van Vliet, 1979). The key provided most detail in the severity range 1-40%, the more relevant part of the total range (0-100%). The assessments were made on a 'middle' leaf level (about leaf 15) and a 'high' leaf level (about leaf 28).

Primary colonies result immediately from inoculation ($1^{st}$ generation), secondary colonies result from primary-lesion spores and appear on noninoculated leaves ($2^{nd}$ generation). Colonies which belong to the 3 and later generations were called tertiary colonies. In Exp-1, the number of secondary and tertiary young mildew colonies (NSC and TSC) was assessed, 4.5 and 6 weeks after the mildew inoculation, at the 'middle' leaf level (about leaf 15) and the fifth leaf of the young shoots, respectively.

**Mycoparasitism and fungicidal effects**

The mildew colonies affected by mycoparasites, oil, or fungicide were assessed weekly on the 'middle' and 'high' leaf levels (Exp-1) or on leaf discs taken from these leaf levels (Exp-2). A symmetrical key with five classes (0 = healthy mildew, 1 = <10%, 2 = 10-35%, 3 = 36-65%, 4 = 66%-90%, 5 = >90% of mildew affected), modified after Philipp *et al.* (1984), was used to classify the percentage of mildew visibly affected by mycoparasites, oil, or fungicide.

Presence of mycoparasites on mildew colonies was determined weekly. In Exp-1, one leaf disc of 2.5 $cm^2$ per plot was punched out a few hours before a new treatment. In Exp-2, the two leaf disks per plot mentioned above were used. Leaf discs were placed on moist filter paper in petri dishes (5 cm diam, one per dish) and were inspected for the presence of *V. lecanii* and/or *S. rugulosa* after 48 h incubation in the dark at 20°C.

**Yield**

From each experimental unit, cucumbers were harvested and weighed twice a week.

**Environmental conditions**

Temperature and humidity in the glasshouse were recorded every ten minutes by sensors coupled to a data logger.

**Statistics and data processing**

*Mildew.* For statistical analyses the mildew assessments (means of the scored classes) were averaged per plot. Observed responses (severities) were rearranged in derived responses (Campbell and Madden, 1990), the area under the mildew progress curve (AUMPC), the early growth rate of mildew (EGR) and the maximum mildew levels (MML).

For Exp-1 the AUMPC was determined per plot for assessments 1 through 5. The AUMPC for Exp-2 was determined over the total range of assessments. The analysis of variance was applied to square roots of these data to improve homogeneity of error variances. For the comparison of means the Tukey test was used.

EGRs were calculated by linear regression through three data points per plot, Julian days 141, 147, and 154 in Exp-1, and 273, 280, and 288 in Exp-2. A $\sqrt{(EGR + 0.5)}$ transformation was carried out before analysis of variance. Comparisons of means were made by the Tukey test.

MMLs were determined per plot by taking the means of the last three assessments per experiment. Analysis of variance (ANOVA) and Tukey test were carried out with arcsine square root transformed data.

For the numbers of colonies in Exp-1 a square root transformation was used before ANOVA and Tukey test were carried out.

*Mycoparasitism and fungicidal effects.* The final level of affected mildew (FLA) was determined for Exp-1 and Exp-2 by taking the mean of the last three assessments per plot and per treatment. FLAs were compared by ANOVA and Tukey test. The final level of leaf area infected with healthy mildew (FLH), which represents the ultimate severity of the disease, was calculated by

$$FLH = MML - FLA * MML$$

Arcsin square root transformed FLH data were compared by ANOVA and Tukey test.

To test the independence of treatments in Exp-1, the frequency distribution of mycoparasites over the treatments was tested by the exact-square test by using the program StatXact of CYTEL Software Corporation (Anonymous, 1991d).

*Yield.* Treatment effects on cumulative yield of cucumbers heavier than 400 g per plot per experimental unit were tested by ANOVA and Tukey test.

## Results

### Mildew Progress Curves (MPC)

Fig. 2A shows the MPCs of Exp-1 on the susceptible cucumber cv Corona. One week after the third biocontrol treatment (day 148), *S. rugulosa* did no longer give satisfactory control on the susceptible cucumber cultivar cv Corona, so that this biocontrol treatment had to be replaced by fungicide (fenarimol) to avoid cross-infection between treatments. Similarly, *V. lecanii* was replaced by fungicide one week after the fourth treatment. When Hora was used as an additive replacement of mycoparasites by fungicide was not necessary. Hora alone or with *V. lecanii* gave excellent control, while Hora with *S. rugulosa* did not. The ranking order of treatments on cv Flamingo during Exp-1 (Fig 2B) is roughly as on cv. Corona. *V. lecanii* kept the mildew just below the 15% level. The MPCs of Exp-2 on cv Flamingo showed that Hora gave good, *V. lecanii* modest, and water poor control, whereas treatment once a week gave better control than once a fortnight (data not shown).

### Area Under the Mildew Progress Curve (AUMPC)

The AUMPCs on Corona and Flamingo summarize mildew progress during the first 5 weeks of Exp-1 (Table 2). *V. lecanii* gave significantly better control than *S. rugulosa*. AUMPCs for treatments with mycoparasites and Hora were significantly smaller than those for mycoparasites without Hora. Hora, with or without *V. lecanii*, gave significantly better control than Hora with *S. rugulosa*.

In Exp-2, the Hora treatments gave significantly better control than the *V. lecanii* or water treatments (Table 3). There were no significant differences between the water and *V. lecanii* treatments.

### Early Growth Rates (EGR) of S. fuliginea

On cv Corona in Exp-1, EGRs for mildew with *V. lecanii* were lower than with *S. rugulosa*, both with or without Hora (Table 2). On cv Flamingo these differences were not significant.

In Exp-2, EGRs of weekly treatments were lower, but not significantly lower, than of fortnightly treatments (Table 3). A weekly Hora treatment was significantly better than *V. lecanii* alone and water treatments (weekly or biweekly). The EGR of a weekly *V. lecanii* treatment was significantly smaller than the EGR of the biweekly water treatment. When we omitted the Hora control treatment, weekly treatments delayed the mildew epidemic significantly ($P \leq 0.05$) more than

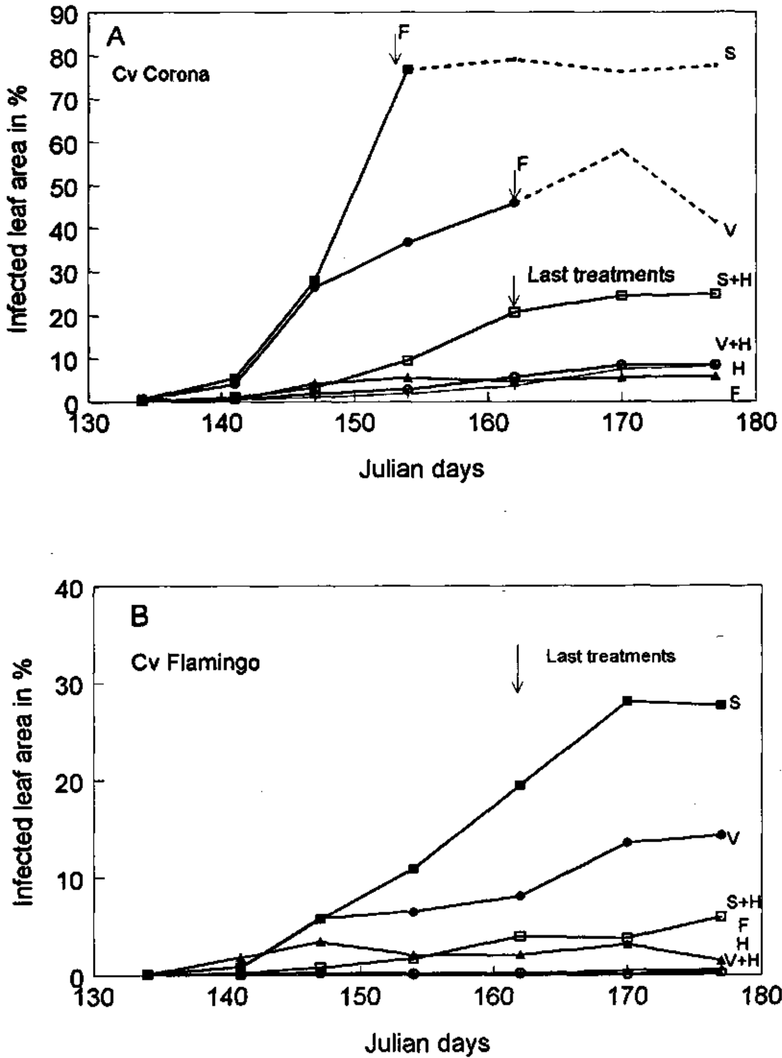


Fig. 2. Experiment 1. Mildew progress curves on cvs Corona (A) and Flamingo (B). Inoculation with *Sphaerotheca fuliginea*, the mildew pathogen, took place on day 112. The weekly control treatments, ■ = *Sporothrix rugulosa* (S), ● = *Verticillium lecanii* (V), □ = S+Hora Oleo 11E (H), O = V+H, and + = H were applied on days 128, 135, 142, 148, 155, and 163, while the fungicide treatment (▲ = F) was applied on days 133, 152, and 164. Entries are averages of six replicates, each replicate representing the mean of six leaves (two leaves of each of the plants). After some time, the S and V treatments on Corona (Fig 2A) were replaced by a fungicide treatment (arrow , ↓F).

the fortnightly treatments, while the *V. lecanii* treatments delayed the mildew epidemic significantly ($P \leq 0.05$) more than the water treatments.

**Maximum Mildew Level (MML)**

*V. lecanii* gave a significantly lower MML than *S. rugulosa* (Table 2). The MML for treatments with mycoparasites and Hora were significantly smaller than those for mycoparasites without Hora. Hora with or without *V. lecanii*, gave significantly lower MML than Hora with *S. rugulosa.*

In Exp-2, MMLs in Hora treatments were significantly lower than in other treatments (Table 3).

**Number of Secondary and Tertiary Colonies (NSC and NTC)**

Very few primary infections were observed on noninoculated leaves. In Exp-1, NSCs on cv Corona with Hora, alone or as an additive, and fungicide treatments were significantly lower than with treatments consisting of the mycoparasites alone (Table 4). A significantly lower amount of NSCs was formed on *V. lecanii*- than on *S. rugulosa*-treated plants. Cv Flamingo showed the same pattern but differences were small. The NTCs on cv Corona were suppressed most by the treatments with fungicide, Hora, and *V. lecanii* plus Hora. On cv Flamingo a significantly lower amount of colonies was formed on *V. lecanii*- than on *S. rugulosa*- treated plants. On both cultivars the NTCs were significantly more suppressed by *V. lecanii* with Hora than by *S. rugulosa* with Hora.

**Yield**

The main yield was about 12 kg per plant, a few kg less than commercial yields, which can be explained by the mildew epidemic and the short duration of the cultivation. Exp-1 and Exp-2 gave no significant differences in yield between cultivars and treatments (data not shown).

**Mycoparasitism and fungicidal effects**

On cv Flamingo, Hora, with or without mycoparasites, gave the lowest levels of percentage affected mildew (FLA) and the mycoparasites gave the largest FLA (Table 5). On cv Corona treatments with *S. rugulosa* and *V. lecanii* showed low levels of FLA before they had to be replaced by fungicide treatments. On both cultivars the final level of the percentage leaf area infected with healthy mildew (FLH) was significantly smaller after *V. lecanii* with Hora treatment than after *S.*

Table 2. Experiment 1. Area Under the Mildew Progress Curves (AUMPCs), Early Growth Rates of mildew (EGRs) and Maximum Mildew Levels (MMLs) on cvs Corona and Flamingo.

| | Corona | | | Flamingo | | |
|---|---|---|---|---|---|---|
| Treatment[a] | AUMPC[b] | EGR[c] | MML[d] | AUMPC[b] | EGR[c] | MML[d] |
| S | 1118 d[e] | 5.53 d | 77.7 d[f] | 202 d | 0.78 c | 25.1 d |
| V | 665 c | 2.49 c | 48.5 c[f] | 117 cd | 0.43 bc | 12.0 c |
| SH | 185 b | 0.67 b | 23.3 b | 35 ab | 0.11 ab | 4.6 b |
| VH | 39 a | 0.06 a | 4.7 a | 5 a | 0.01 a | 0.2 a |
| H | 47 a | 0.10 a | 6.4 a | 5 a | 0.00 a | 0.4 ab |
| F | 96 ab | 0.37 ab | 5.4 a | 57 bc | 0.01 a | 2.2 ab |

[a] For treatment codes, see Table 1.
[b] AUMPCs data were transformed to square roots before analysis.
[c] EGRs (% infected leaf area/day) data were transformed to $\sqrt{(EGR+0.5)}$ before analysis.
[d] MMLs (% infected leaf area) data were transformed to arcsine square roots before analysis.
[e] Means within a column with the same letter are not significantly different according to the Tukey Multiple Range test ($\underline{P} \leq 0.05$).
[f] Replaced by fungicide, S at day 153 and V at day 163.

Table 3. Experiment 2. Area Under the Mildew Progress Curves (AUMPC's), Early Growth Rates of Mildew (EGRs) and Maximum Mildew Levels (MMLs) on cv. Flamingo.

| Treatments[a] | AUMPC[b] | EGR[c] | MML[d] |
|---|---|---|---|
| V1 | 833 b[e] | 1.42 bc | 28.3 b |
| V2 | 969 b | 4.80 cd | 31.2 b |
| W1 | 882 b | 6.87 cd | 30.7 b |
| W2 | 1171 b | 12.94 d | 46.5 b |
| H1 | 15 a | 0.09 a | 0.3 a |
| H2 | 114 a | 0.68 ab | 4.4 a |

[a] For treatment codes, see Table 1.
[b] AUMPCs data were transformed to square roots before analysis.
[c] EGRs (% infected leaf area/day) data were transformed to $\sqrt{(EGR+0.5)}$ before analysis.
[d] MMLs (% infected leaf area) data were transformed to arcsine square roots before analysis.
[e] Means within a column with the same letter are not significantly different according to the Tukey Multiple Range test ($P \leq 0.05$).

*rugulosa* with Hora treatment. Exp-2 gave no significant differences in FLAs (data not shown).

Based on the presence of mycoparasites on the leaf discs during Exp-1, the hypothesis of independence between the treatments could not be rejected at the 5% level of significance for the first 4 weeks according to the exact-square test. Thus, we consider the treatments to be independent, at least during the first months following Julian day 135.

**Environmental conditions**

Fig. 3 shows the temperature of days and nights, and relative humidity for Exp-1. The long-term means for temperature and relative humidity of Exp-2 were similar to those of Exp-1 but the ranges between day and night values were much smaller.

## Discussion

**Experiment 1**

On both cucumber cultivars, *V. lecanii* reduced the mildew considerably in comparison to *S. rugulosa.* On cv Flamingo *V. lecanii* could keep the mildew below 15% infected leaf area. In preliminary experiments we observed that mildew colonies on Corona grew about twice faster and formed more conidiophores per $mm^2$ than on Flamingo. Abul-Hayja (1982) found that 16 days after inoculation the mildew produced about 40 times more conidia on susceptible leaves than on partially resistant leaves. We assume that mycoparasites can more easily destroy slow than fast growing mildew colonies.

Various mechanisms may explain the fact that Hora Oleo 11E controlled the mildew at least equally well without as with mycoparasites. Firstly, oils can damage fungi. Ohtsuka and Nakazawa (1991) found that a film of machine oil caused deformation of conidia of cucumber powdery mildew. Secondly, oils can change the plant's physiology. Leaves treated with oils may contain more sugars because of the decrease in assimilation, which could inhibit mildew development (Horsfall and Dimond, 1957). Severity of cucumber powdery mildew was reduced by spraying Canola oil and Agral 90 (Schneider and Northover, 1991). Plant oils were effective against apple powdery mildew without much specificity attributable to chemical structure. Chemically distinct oils might act similarly by altering the physiology of plant-host relationship (Northover and Schneider, 1993). Thirdly, oils influence the microflora on the leaves. Philipp *et al.* (1990), who tested different formulations of the mycoparasite *Ampelomyces quisqualis* Ces. on cucumber powdery mildew,

Table 4. Experiment 1. Number of Secondary (NSC) and Number of Tertiary mildew (NTC) Colonies on Corona and Flamingo plants after different treatments.

| | Corona | | Flamingo | |
|---|---|---|---|---|
| Treatments[a] | NSC[b] | NTC[b] | NSC | NTC |
| S | 174 c[c] | 124 d | 18.1 c | 87 c |
| V | 81 b | 118 d | 8.3 bc | 30 ab |
| SH | 13 a | 76 cd | 0.4 a | 59 bc |
| VH | 5 a | 37 b | 0 a | 12 a |
| H | 3 a | 42 bc | 2.2 ab | 35 ab |
| F | 6 a | 6 a | 11.8 bc | 19 ab |

[a] For treatment codes, see Table 1.
[b] NSC and NTC data (mean numbers of secondary and tertiary mildew colonies) were transformed to square roots before analysis.
[c] Means within a column with the same letter are not significantly different according to the Tukey Multiple Range test ($P \leq 0.05$).

Table 5. Experiment 1. The Final Levels of percentages Affected mildew (FLAs) and Healthy mildew (FLHs) on cvs Corona and Flamingo after different treatments.

| | Corona | | Flamingo | |
|---|---|---|---|---|
| Treatments[a] | FLA[b] | FLH[c] | FLA[b] | FLH[c] |
| S | -[d] | - | 91.1 c | 2.4 c |
| V | - | - | 76.7 c | 2.8 c |
| SH | 51.6 a[e] | 11.8 b | 51.2 b | 1.8 bc |
| VH | 61.6 a | 1.3 a | 14.1 a | 0.2 a |
| H | 60.2 a | 2.2 a | 12.3 a | 0.3 ab |
| F | 68.3 a | 1.4 a | 51.6 b | 1.0 abc |

[a] For treatment codes, see Table 1.
[b] FLA (Final Level of % affected mildew).
[c] FLH (Final Level of % leaf area infected with Healthy mildew), data were transformed to arcsine square roots before analysis.
[d] Replaced by fungicide, S at day 153 and V at day 163.
[e] Means within a column with the same letter are not significantly different according to the Tukey Multiple Range test ($P \leq 0.05$).

concluded that Hora Oleo 11E was most effective by reducing the humidity demand of *A. quisqualis*, though parasitization of powdery mildew by *A. quisqualis* decreased when Hora Oleo 11E was added.

Since the percentage of affected mildew on cv Flamingo was higher after treatment with mycoparasites than with either mycoparasites plus Hora, Hora alone, or fungicide, two different control mechanism might play a role. After treatments with mycoparasites plus Hora, Hora may destroy most of the mildew and thus create favourable nutritional conditions for mycoparasites and antagonistic saprophytes. Hora with or without mycoparasites may destroy mildew within a week while mycoparasites without Hora perform more slowly. Consequently, a week after spraying, the treatment with Hora only will carry few but mainly healthy mildew colonies, either missed by the treatments or younger than one week and not yet treated. On cv Corona the total amounts of affected mildew after Hora treatments, with or without *V. lecanii,* was larger than on cv Flamingo. This might be explained by the fact that destroyed mildew colonies remained better visible on the susceptible cv Corona than on the partially resistant cv Flamingo. Although *S. rugulosa* with Hora showed a higher percentage affected mildew than Hora alone or *V. lecanii* with Hora, the total amount of infected leaf area with healthy mildew was significantly larger on plants treated with *S. rugulosa* with Hora.

Bélanger *et al.* (1994) reported good control of *Sphaerotheca pannosa* (Wallr.:Fr.) Lév. var. *rosae* in glasshouse experiments by *Sporothrix flocculosa* Traquair, Shaw and Jarvis with 1% paraffin oil. As they did not test the paraffin oil alone, it is not clear whether the mycoparasite, the oil, or the combination was responsible for the good control.

**Experiment 2**

Results of the AUMPC and MDL do not permit to reject the hypothesis that the weekly and fortnightly treatments are equal in the cases of *V. lecanii* and water. The significant difference in EGR between the fortnightly water and the weekly *V. lecanii* treatments indicates that differences between the two treatments are not large enough to be detected by AUMPC and MDL with the given method and sample size. *Verticillium lecanii* was observed on leaf discs of every treatment. The mean frequencies of *V. lecanii* in weekly treatments were V1, 82%; W1, 56%, and H1,36%. The fortnightly treatments showed the same order: V2, 74%; W2, 60%, and H2, 44%. Apparently, part of the control on the Hora and water treatments was

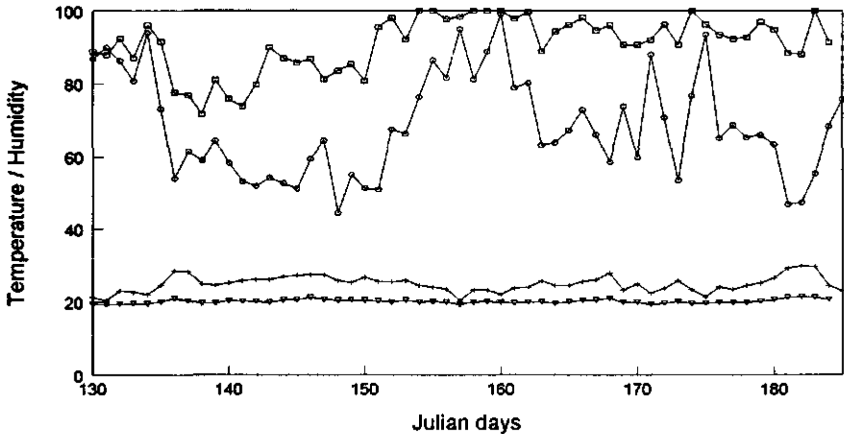


Fig. 3. Mean temperatures and relative humidities of days and nights of experiment 1 (□ = night humidity, O = day humidity, + = day temperature, ▿ = night temperature).

due to biological control, possibly after cross contamination by *V. lecanii*. It seems better to isolate experimental units during spraying by plastic curtains, as in Exp-1.

**General remarks**

In both experiments biological control began 1 week after the first observation of mildew on the inoculated leaves. The mildew was given 1 week to develop without any control treatments. An earlier start of biological control might have given better results.

With hindsight, it would have been better to use 0.1% Hora instead of 0.5%. A lower concentration of Hora might have had a smaller effect on the mildew by itself (Hijwegen, 1992). The phyllosphere is a complex system. Apart from the pathogen various other organisms may thrive on the leaf and interfere in the proces of mildew development. A major change, such as the application of Hora and/or mycoparasites, will profoundly influence all the organisms in the system. Hora may create new opportunities for the sedentary microorganisms to antagonise the mildew. On many occasions bacteria deleterious to powdery mildew were isolated. The ubiquitous fungi *Cladosporium sphaerospermum* and *Penicillium* spp. were

abundantly present on older mildew colonies not treated by mycoparasites. These fungi are weakly antagonistic to *S. fuliginea*. We hypothesize that treatment with mycoparasites may somehow antagonize these weak bacterial and fungal antagonists. In other words, the antagonist may be antagonized and the net effect can be next to nil (Hijwegen, 1992). This may explain that the performance of *S. rugulosa* with Hora was poorer than that of Hora alone.

These glasshouse experiments demonstrated that biological control of *S. fuliginea* by *V. lecanii* in combination with the use of partially resistant cucumber cultivars has prospects. Selection for *V.lecanii* isolates with a higher control potential under glasshouse conditions might improve the biological control. In addition, a search for formulations of biological control agents is important because formulations can improve the efficiency of the mycoparasites, especially under low humidity conditions. Care is needed since, in The Netherlands, formulation chemicals tend to be seen as pollutants and thus have to be screened before the formulated mycoparasite can be registered.

# Chapter 5

# Selection of *Verticillium lecanii* isolates with high potential for biocontrol of cucumber powdery mildew by means of components analysis at different humidity regimes

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# Selection of *Verticillium lecanii* isolates with high potential for biocontrol of cucumber powdery mildew by means of components analysis at different humidity regimes

## Abstract

To identify characteristics for the selection of *Verticillium lecanii* isolates with high potential for biocontrol of *Sphaerotheca fuliginea* under glasshouse conditions, an exploratory study was performed on the effect of water limitation on the development of 14 isolates. Conidial germination, growth and sporulation of isolates of *V. lecanii* were studied in a tritrophic system on cucumber leaves and in a ditrophic system in Petri dishes. Their mycoparasitic ability was studied on *S. fuliginea* and *Cladosporium cladosporioides*. All characteristics were clearly affected by humidity. Four isolates showed good biocontrol potential. The performance of isolates on agar had less predictive value than on powdery mildew. Germination of isolates of *V. lecanii* was lower and mycelial growth faster on agar than on mildewed leaves under corresponding humidity conditions. The results suggest that conditions in the phyllosphere differed from the set humidity in the surrounding air. A correlation was found between lysis of *C. cladosporioides* growing in dual culture on agar with isolates of *V. lecanii* and parasitism of powdery mildew on detached, rooted leaves. *C. cladosporioides* might offer a suitable substrate for testing isolates of *V. lecanii* for mycoparasitic potential at various environmental conditions. Conidial germination, growth and sporulation had limited predictive value.

## Introduction

The mycoparasite, *Verticillium lecanii* (Zimm.) Viégas, is a promising biological control agent of *Sphaerotheca fuliginea* (Schlecht.:Fr.) Poll., especially in combination with partial resistance (Verhaar *et al.*, 1996). *V. lecanii* grows over a wide temperature range (Magan & Lacey, 1984) and a water film, or at least high humidity, is required for conidial germination (Hall, 1981a). Thus, water is an important environmental factor in the biocontrol of powdery mildew on cucumber and rose by *V. lecanii* (Bélanger *et al.*, 1994, Verhaar *et al.*, 1996). Development of *V. lecanii* in pustules of *Puccinia striiformis* was best at 95 to 100% relative humidity (RH), whereas at 80% RH no development was observed (Mendgen,

1981). The effectiveness of *V. lecanii* as an entomopathogen is influenced by humidity (Drummond *et al.*, 1987). High humidities were needed for high infection levels of aphids (Milner & Lutton, 1986) and greenhouse whiteflies (Ekbom, 1981), but Hsiao *et al.* (1992) found 100% mortality of aphids treated with *V. lecanii* at 76% RH.

In Dutch glasshouses, the relative humidity of the ambient air varies between 65 and 95%. A RH $\geq$ 80% occurs daily for 8 to 12 h at night. Less than optimum humidity, even at night, could limit the development of *V. lecanii*. Selection for isolates of *V. lecanii* that can tolerate limiting humidity conditions would improve biological control of powdery mildew by *V. lecanii* in a glasshouse environment.

The objective of this study was to determine the effect of water limitation on development of isolates of *V. lecanii*. The development of *V. lecanii* was subdivided into the components: germination, growth, sporulation and mycoparasitism as in components analysis (Zadoks & Schein, 1979). Mycoparasitism was studied on powdery mildew growing on cucumber leaves, and on *Cladosporium cladosporioides* (Fresen.) de Vries on agar in Petri dishes. Correlation between mycoparasitic potential on mildew and sensitivity to water deficit of various components in the life cycle of *V. lecanii* as well as parasitism on *C. cladosporioides* were examined.

## Materials and methods

### The mycoparasite

Strains of *V. lecanii* were isolated from foliar pathogens, mostly powdery mildews in the Netherlands and Germany (Table 1). Isolates were stored by freezing conidial suspensions in 10% glucose solution in 1 ml plastic ampoules at -80°C. Isolates were cultured on oatmeal agar (60 gr milled oatmeal (HO, Quaker) and 20 gr agar (Technical No.3, Oxoid) in 1 l water) plates and maintained at 20°C in the dark. Conidia were harvested by flooding 8-10 day-old cultures with 10 ml of distilled water. Suspensions were filtered through cotton wool and adjusted to 5 x $10^6$ conidia/ml using a haemacytometer for use in experiments. Development of *V. lecanii* on mildewed cucumber (*Cucumis sativus* L.) leaves and on *C. cladosporioides* was observed using a stereomicroscope with continuous variable magnification (8 to 50x).

Table 1. Isolates of *V. lecanii* used in this study

| Isolate | Year of isolation | Origin |
|---|---|---|
| 1 | 1988 | ex *Sphaerotheca fuliginea* on *Cucumis*, Wageningen, NL (Fyto 88.1) |
| 2 | - | Mycotal isolate (Koppert BV, Berkel en Rodenrijs, NL) |
| 3 | 1973 | IMI 179173 ex *Brachycaudus helichrysi* |
| 4 | 1973 | CBS 470.73 ex *Hemileia vastatrix* on *Coffea*, India |
| 5 | 1993 | ex *Uncinula* on *Salix*, Walporzheim, Germany |
| 6 | 1993 | ex *Uncinula* on *Vitis*, Ahrtal, Germany |
| 7 | 1993 | ex *Uncinula* on *Vitis*, Ahrtal, Germany |
| 8 | 1993 | ex *Botrytis* on *Gladiolus*, Bennekom, NL |
| 9 | 1993 | ex *Botrytis* on *Gladiolus*, Wageningen, NL |
| 10 | 1993 | ex *Bremia* on *Lactuca*, Wageningen, NL |
| 11 | 1993 | ex *Uncinula* on *Acer*, Ahrweiler, Germany |
| 12 | 1993 | ex *Peronospora* on *Brassica*, Wageningen, NL |
| 13 | 1993 | ex *Erysiphe* on *Hordeum*, Wageningen, NL |
| 14 | 1993 | ex *Microsphaera* on *Mahonia*, Marienthal, Germany |

**Experiments on mildewed leaves**

*Cucumber*. Cucumber plants (cv. Corona, De Ruiter Seeds, Bleiswijk, The Netherlands) were grown in a growth chamber (20°C; 16 h light, Pope FTL D32W/84HF; 70% RH). Rooted leaves were prepared by excising second and third leaves of 3-week-old plants and placing them through a hole between two, glued together 9-cm plastic Petri dishes, with the petiole in the lower dish containing 50% Hoagland's solution as described by Verhaar *et al.* (1997). The petioles produced roots in the lower dish in about 7 days.

Colony growth, mycoparasitism and germination of *V. lecanii* were observed on leaf disks or rooted cucumber leaves incubated in plastic boxes (30 x 45 cm, 30 cm high) with controlled humidities. A near-saturated to saturated atmosphere (95-100% RH) was maintained by 1 l of water at the bottom of individual boxes; reduced humidities were maintained by a saturated salt (NaCl) solution (Winston & Bates, 1960). The relative humidities were set using a thermohygrometer (Rotronic Ltd, Germany), with a nominal accuracy of ± 0.1°C and ± 0.5% RH. In boxes with respiring leaves, a saturated solution of NaCl, which usually gives approximately 75% RH, resulted in approximately 85% RH. The boxes were incubated in a growth chamber (20°C; 16 h light, 23 W $m^{-2}$ at leaf level, Pope FTL D32W/84HF; 70% RH). During the experiments, humidities in the boxes were controlled by small LCD digital thermohygrometers (Omnilabo Ltd, Breda, The Netherlands) placed in the boxes (accuracy ± 1°C and ± 7% RH).

*Experiment 1. Colony growth of* V. lecanii. Detached and rooted leaves (Verhaar *et al.*, 1997) were inoculated with cucumber powdery mildew in a vacuum-operated settling tower (Reifschneider & Boiteux, 1988). By interrupting a vacuum of -15 kPa, conidia were detached from a rooted cucumber leaf with mildew and evenly distributed over 24 Petri dishes. After a settling time of 2 min, the Petri dishes were closed and incubated in the growth chamber.

Five days after inoculation with *S. fuliginea*, the mildewed leaves were inoculated with *V. lecanii*. Droplets of 0.02 ml (5 x $10^6$ conidia/ml) were placed on the mildewed leaves. On each mildewed leaf, three different isolates of *V. lecanii* were tested with a distance of 3.5 cm between the droplets. Open Petri dish units containing mildewed leaves inoculated with *V. lecanii* were placed on grids in boxes containing water or salt solution. The boxes were closed to maintain the desired humidities. Colony diameters of *V. lecanii* developing on the mildew were measured four times at intervals of 2 or 3 days. Each isolate was tested on four mildewed leaves and the mean diameter/isolate was calculated for each observation day. Radial colony growth rates of *V. lecanii* were determined from the gradients of the fitted regression lines for colony diameter against time.

*Experiment 2. Mycoparasitism on* S. fuliginea *and secondary mildew colonies.* This experiment was designed to test the survival of mildew after mycoparasitism by *V. lecanii*. Survival was assessed as the number of second generation mildew colonies initiated on fresh leaves by mildew conidia taken from mycoparasitized colonies. The first part of the experiment resembled experiment 1, except that conidial

suspensions of *V. lecanii* and water as a control, were sprayed over mildewed leaves by means of a De Vilbiss sprayer. One isolate/leaf and three replicates/isolate-humidity combination were used. This part of the experiment had a randomized complete block design with three replicates. The percentage of mildewed area/leaf colonized by *V. lecanii* was assessed 7 days after biocontrol treatment.

To assess the infectivity of parasitized mildew one week after biocontrol treatments, a leaf disk (20mm diameter) was cut from the centre of each leaf. Three leaf disks/isolate-humidity combination were bulked, and used to inoculate three fresh cucumber plants as described before. Inoculated plants were placed randomly in a glasshouse compartment (about 20°C and 70% RH). One week later, leaf lengths and numbers of mildew colonies/leaf were assessed on three leaves/plant (cv. Corona). The leaf area was estimated using an equation: $A = 46.20 - 10.14L + 1.26L^2$ ($R^2$=0.95), where A = leaf area and L = measured leaf length (derived from a preliminary experiment (Verhaar, unpublished)). Colony counts/leaf were converted to numbers of colonies/$dm^2$.

*Experiment 3. Conidial germination* of V. lecanii. Conidial suspensions ($5x10^6$ conidia/ml) of nine isolates of *V. lecanii* were sprayed on cucumber leaf disks (20 mm diameter) laid on water agar in Petri dishes. Dishes were incubated in plastic boxes with either a near-saturated to saturated atmosphere of about 85% RH as described above, or in open boxes in the growth chamber (about 65% RH). After 24 h of incubation in the growth chamber, conidia were killed by placing the Petri dishes in a desiccator with 40% formaldehyde for 5 min. The phylloplane microflora was stripped from the leaf surfaces with collodion. Germination of at least 100 conidia/leaf disk was observed using a microscope (x100 magnification). A conidium was considered to have germinated when the length of the germ-tube was at least equal to the smallest diameter of the conidium.

**Experiments on agar plates**

Growth, sporulation, germination and mycoparasitism were assessed on agar in 9 cm diameter Petri dishes with water potentials adjusted over the range of -9.8 to -0.5 MPa by addition of NaCl (Lang, 1967). To compare RHs in the tritrophic experiments with water potentials in the ditrophic experiments, water potentials of -0.5, -5.6, -7, -8.4 and -9.8 MPa, equivalent to approximately 99.5, 96, 95, 94 and 93% RH at 20°C (Papendick & Campbell, 1981), were chosen. The water potential

of all media was checked by a thermocouple psychrometer before use (Decagon Devices Inc, Pullman, WA, USA).

*Experiment 4. Growth of isolates of* V. lecanii *on agar.* Plates of malt extract agar (Oxoid CM59) adjusted to -0.5 and -7 MPa were inoculated centrally with a 5mm diameter mycelium disk, and radial growth rate at 20°C was determined. Colony diameters were assessed on four replicates/isolate x humidity combination, 6, 9 and 13 days after inoculation.

*Experiment 5. Conidial germination of* V. lecanii *on agar.* Five isolates of *V. lecanii* were chosen by selecting two isolates with germination ≥ 65% (isolates 1 and 12), and three isolates with germination of about 40% (isolates 4, 5 and 14) at 85% RH from experiment 3. Droplets (10µL, $10^4$ conidia) were placed on water agar (WA) plates with water potentials of -0.5, -5.6, -7, -8.4 and -9.8 MPa, with one droplet/isolate/plate, and six plates/water potential. Droplets were air-dried for 2 h in a laminar flow cabinet. The Petri dishes were closed and incubated for 22 h at 20°C in the dark. Percentage germination was determined by assessing 100 conidia/droplet as described before.

*Experiment 6. Mycoparasitism on and lysis of* C. cladosporioides. Plates of WA were adjusted to -0.5 and -7 MPa. Using an inoculation needle, each plate was point-inoculated with *C. cladosporioides* 3 cm from the edge. Two days later, one of 14 *V. lecanii* isolates was point-inoculated on each plate at a position 3 cm from the edge, and 2 cm from the inoculation point of *C. cladosporioides*. Petri dishes were incubated at 20°C under diffuse daylight for 12 h/day. Percentage parasitism and lysis of hyphae of *C. cladosporioides* was assessed after 40 days using a scale of 0-5 where 0 = 0%, 1 = 1-20%, 2 = 21-50, 3 = 51-80%, 4 = 81-99% and 5 = 100% parasitism. Each *C. cladosporiodes* x *V. lecanii* x water potential combination was replicated four times.

*Experiment 7. Sporulation of* V. lecanii. Sporulation of *V. lecanii* isolates after 40 days growth on WA adjusted to -0.5 and -7 MPa was assessed by using a sporulation score of 0-5, where 0 = no sporulation and 5 = abundant sporulation.

**Experimental design and statistical analyses**

Experiments were performed twice (experiments 2, 3 and 6) or three times (experiment 1, 4, 5 and 7), with a minimum of three replicates/treatment. Data on

growth, sporulation and germination were analyzed as a mixed model with water potential or relative humidity as fixed vectors, and isolate as the stochastic vector (Scheffé, 1959). Data were tested for normality and heterogeneity of variance. Percentage parasitized mildew, number of secondary colonies in experiment 2, and percentage germinated conidia in experiment 3 were arcsine transformed before ANOVA to improve homogeneity of variance. Data from each experiment were subjected to ANOVA. To study the consistency of the sequential experiments, combined ANOVAs (Gomez & Gomez, 1984) were performed over the replicate experiments (indicated as 'blocks'). Data from water controls of growth experiments were included in the figures. Unless stated otherwise, they were excluded from ANOVAs as the data were irrelevant to the comparison of isolates. Isolates were compared with isolate 1, an isolate that was used in previous experiments (Verhaar *et al.*, 1996; 1997), using the least significant difference (LSD).

Correlations between parasitized mildewed leaf area and number of second generation powdery mildew colonies, conidial germination, colony growth and sporulation of *V. lecanii* isolates were determined by Spearman Rank correlations.

Germination data of experiment 5 were subjected to a probit analysis by plotting the probits of germination percentages against water potentials. The mid-values (here the water potential at 50% germination) and slopes of the probit lines were subjected to ANOVA.

## Results

*Experiment 1. Colony growth of* V. lecanii *on mildew.* The means of three replicate experiments (blocks) showed a significant ($P \leq 0.05$) effect of humidity on colony growth rates. *V. lecanii* colonies grew about two times faster at near-saturated to saturated humidity than at a RH of about 85% (Figure 1). The isolates were ranked according to decreasing colony radial growth rates at saturated humidity, clearly showing the difference in the effect of saturated and reduced humidities on colony growth rates. For comparison, the same ranking was used in the figures of all experiments. At very high RH, differences between isolates were considerable, with isolates 4 and 14 being the fastest growers. At reduced RH (about 85%), differences between isolates were relatively small. The combined ANOVA pointed to significant ($P \leq 0.05$) block, humidity and isolate effects (Table 2), and to significant two-way interactions between these factors. The analysis was simplified by

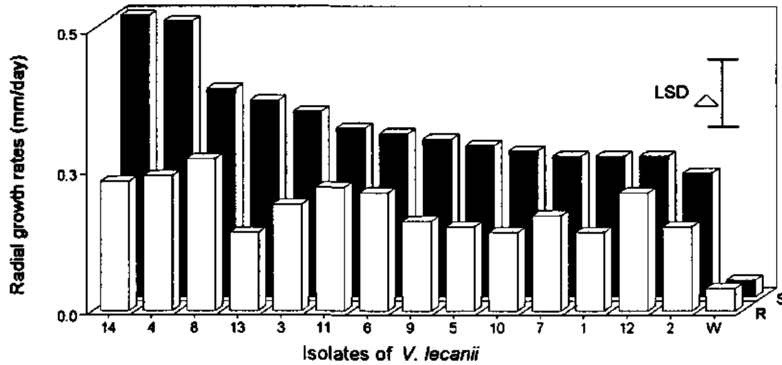


Figure 1. Mean growth rates of 14 *V. lecanii* isolates on mildewed cucumber leaves at near-saturated to saturated (S) RH and reduced RH of about 85% (R). Water (W) without conidia served as the control. Error bar represents the $LSD_{0.05}$ =0.14 (n=12).

Table 2. Experiment 1. Combined ANOVA for growth rates of isolates *V. lecanii* on *S. fuliginea*.

| Source of variation [a] | Degrees of freedom | Sum of squares | Mean square | F-ratio [b] |
|---|---|---|---|---|
| I | 13 | 0.24 | 0.02 | 5.71 ** |
| H | 1 | 0.26 | 0.26 | 78.72 ** |
| R | 2 | 0.58 | 0.29 | 87.70 ** |
| I x H | 13 | 0.12 | 0.01 | 2.70 * |
| I x R | 2 | 0.05 | 0.02 | 6.88 ** |
| H x R | 26 | 0.27 | 0.01 | 3.06 ** |
| Residual | 26 | 0.09 | 0.00 | |
| Total | 83 | 1.62 | | |

[a] I = isolate, H = humidity, R = replicate experiment (block)
[b] ** significant at $P \leq 0.01$, * significant at $P \leq 0.05$

considering only the difference in colony growth rates at the two humidities. The combined ANOVA for differences in growth showed significant ($P \leq 0.05$) block and isolate effects, but no significant interaction between these factors. The original ranking of the isolates was roughly maintained, with isolates 4 and 14 showing a significantly ($P \leq 0.05$) larger difference in growth between the two humidities than isolate 1.

*Experiment 2. Mycoparasitism on* S. fuliginea *and secondary mildew colonies.* Twelve days after mildew inoculation, variation in mildew severity was low among humidity treatments and isolates (Figure 2.IA and 2.IIA). Combined ANOVA for mildew severity yielded significant ($P \leq 0.05$) differences between blocks, but not between humidities and isolates. In block I, isolates 4 and 14 produced abundant mycoparasitism, whereas the uninoculated controls had no mycoparasitism (Figure 2.IB and 2.IIB). Combined ANOVA for percentage parasitized mildew showed significant ($P \leq 0.05$) block, humidity and isolate effects. The block x humidity interaction was significant ($P \leq 0.05$). The significant block effect primarily reflected differences in mildew severities, on average 87% in block I and 38% in block II. Figures 2.I and 2.II suggest an inverse relationship between mildew severity and mycoparasitism; mycoparasitism was high in block II with low mildew severities. ANOVAs for percentage parasitized mildew performed/block yielded significant humidity and isolate effects. Interaction between humidity and isolate was significant ($P \leq 0.05$) only in block I, mainly caused by isolates 4 and 14.

The effect of humidity treatments on reduction in the number of second generation colonies of powdery mildew was weak, but the effect of previous mycoparasitism was pronounced (Figures 2.IC and 2.IIC). ANOVAs performed for each block yielded significant ($P \leq 0.05$) isolate and humidity effects, and a significant isolate x humidity interaction.

At near-saturated to saturated humidity, the Spearman rank correlations between second generation powdery mildew colonies and percentage of mildewed leaf area parasitized by *V. lecanii* were significant ($P \leq 0.05$). At reduced humidity (about 85% RH), the rank correlation was significant in block II but not in block I, clearly because of the generally low levels of mycoparasitism. Isolates 4 and 14 performed well in both blocks and at both humidities.

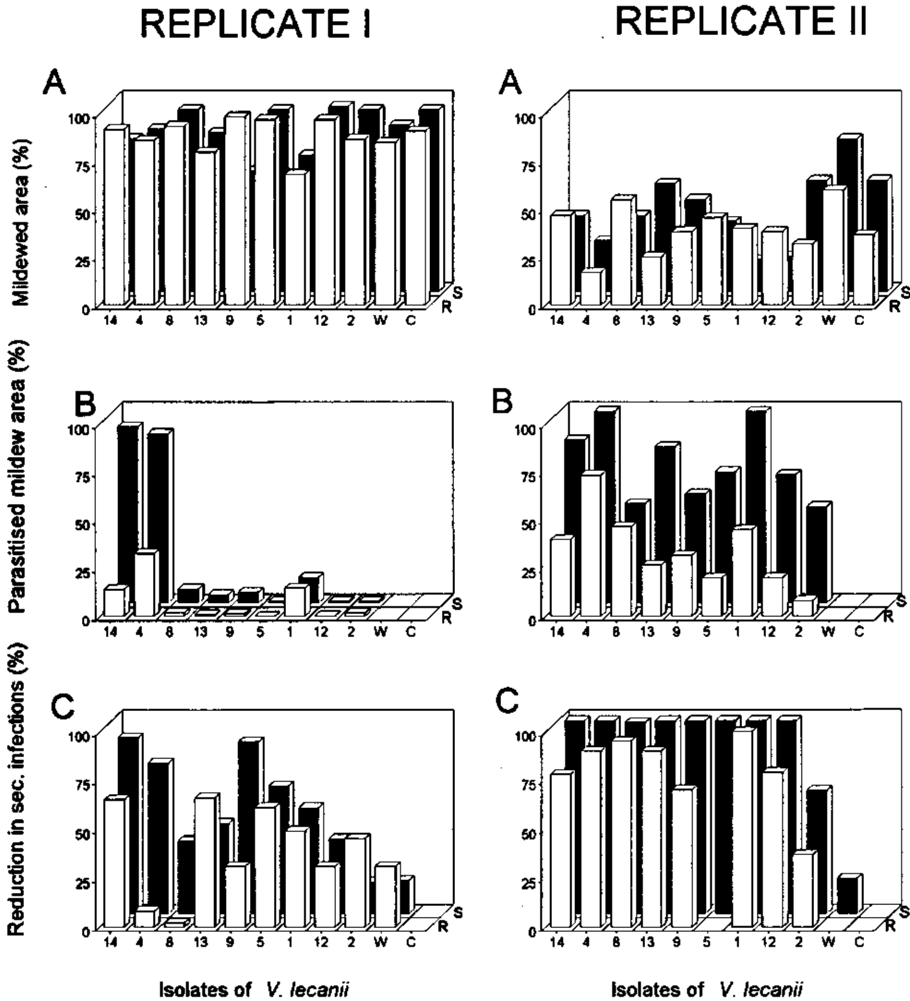


Figure 2. Mean mildewed area (A), mean parasitized mildewed area (B) and mean reduction in secondary infections of *S. fuliginea* (C) after treatment with nine isolates of *V. lecanii* at near-saturated to saturated RH (S) and reduced RH of about 85% (R) (Replicate experiments I and II). Water treated (W) and untreated (C) mildewed cucumber leaves served as controls.

*Experiment 3. Condial germination of* V. lecanii *on cucumber leaves.* Differences between humidity treatments were considerable (Figure 3). Combined ANOVA yielded significant ($P \leq 0.05$) block, humidity and isolate effects. All two- and three-way interactions between these factors were also significant. ANOVA for each block also showed significant humidity and isolate effects. In both blocks, the isolate x humidity interaction was significant ($P \leq 0.002$). This suggests that some isolates tolerate low humidity better than others. Isolate 1 seemed tolerant to reduced humidity (about 85% r.h.) in both blocks, and germinated at about 65% RH in block I but not in block II. Isolates 2 and 9 seemed moderately tolerant to 65% RH in block II but not in block I. The behaviour of isolates 4, 5 and 14 was similar in the two blocks, but these isolates did not germinate well. Generally, differences between humidity treatments were reproducible, but differences between isolates were inconsistent.

*Experiment 4. Radial growth of* V. lecanii *on agar.* At -0.5 MPa, radial colony growth rates (in mm/day) were two to three times higher than at -7 MPa (Figure 4). Differences between water potential treatments were marked and differences between isolates were more pronounced at high than at low water potentials. The combined ANOVA over three blocks showed significant ($P \leq 0.05$) humidity and isolate effects, but no isolate x humidity interaction and no block effect. Isolates 1, 9 and 14 were fast growers. At high water potentials, isolates 4, 7, 8, 11 and 12 had significantly ($P \leq 0.05$) lower radial growth rates than isolate 1, whereas at low water potential, isolates 2, 4, 12 and 13 had lower growth rates than isolate 1.

*Experiment 5. Germination in Petri dishes.* As no significant differences were found between regression coefficients, they were considered to be homogeneous. Differences of 50% germination points between isolates were not significant.

*Experiment 6. Mycoparasitism on and lysis of* C. cladosporioides. Parasitism on *C. cladosporioides* by *V. lecanii* expressed in percentage parasitized colony area was too variable to yield significant isolate effects. The combined ANOVA showed only a significant humidity effect. At -0.5 MPa, most of the isolates parasitized more than twice as much area of the *C. cladosporoides* colonies than at -7 MPa.

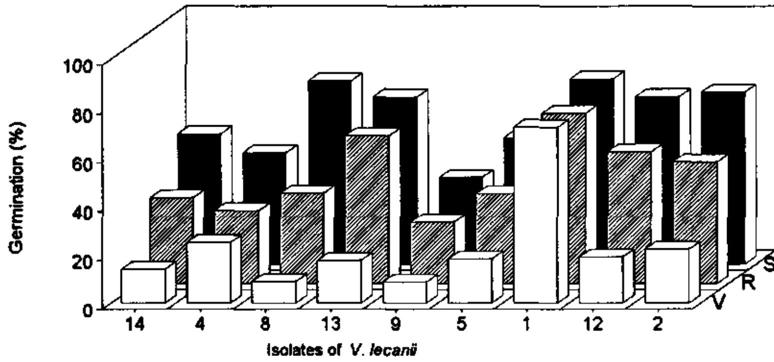


Figure 3. Mean percentage germination of nine isolates of *V. lecanii* on cucumber leaves exposed for 24 h to different RHs, S = near-saturated to saturated, R = reduced (about 85%) and V = 'very' reduced (about 65%) (Replicate experiment I).

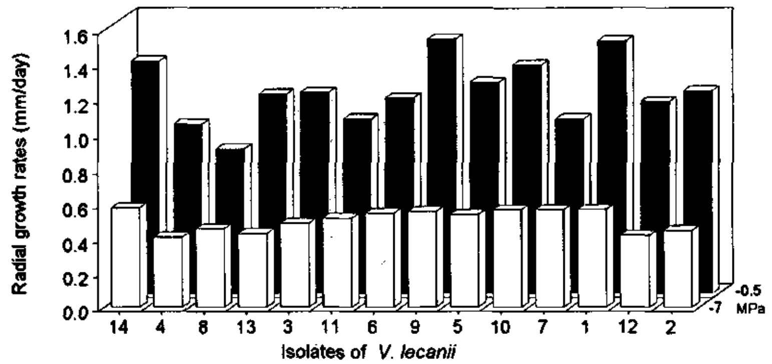


Figure 4. Mean radial growth rates on malt agar of 14 isolates of *V. lecanii* isolates at water potentials of -0.5 MPa and -7 MPa.

The combined ANOVA on percentage lysis of *C. cladosporioides* showed highly significant humidity and isolate effects ($P \leq 0.01$), without block effects or interactions. Interestingly, lysis was often more pronounced at low humidity. Isolates 1, 4, 10, 12 and 14 produced relatively high lysis values at either humidity (Figure 5). Lysis of *C. cladosporioides* at -0.5 MPa was significantly and positively correlated to parasitism of *V. lecanii* isolates on mildew at saturated humidity (experiment 2), with a Spearman rank correlation coefficient of 0.90 ($P \leq 0.05$, n=9). At reduced humidity, the positive correlation of 0.50 was not significant ($P$=0.15, n=9).

*Experiment 7. Sporulation of isolates of* V. lecanii. The ranking order of isolates for sporulation (Figure 6) was similar to that obtained for colony growth on mildew (Figure 1), with the notable exception of isolate 13. The combined ANOVA showed significant ($P \leq 0.05$) isolate and water potential effects (Table 3), and the isolate effect was obvious (Figure 6). The isolate x humidity interaction was significant ($P \leq 0.05$; Figure 6) and humidity had opposite effects on, for example, isolates 1 and 9.

## Discussion

This paper reports exploratory research into differences in mycoparasitic behaviour among isolates of *V. lecanii* by applying components analysis. Systematic comparison of the performances of individual isolates is essential in selecting the best one for biological control and devising predictive laboratory tests. Unfortunately, the characters tested so far seem to have a limited predictive value.

*Colony growth rate of* V. lecanii. Isolates were ranked according to their colony growth rate on mildew, since it was suspected that this character was correlated to mycoparasitic ability of the isolates. The slow growth rates when droplets of water (control treatment) were placed on mildewed leaves can be explained by some cross-contamination by *V. lecanii*. No correlation was found between growth rates of *V. lecanii* isolates on mildew and agar. Colonies of *V. lecanii* isolates on agar of -0.5 MPa grew about three times faster than on mildew at near-saturated to saturated humidity.

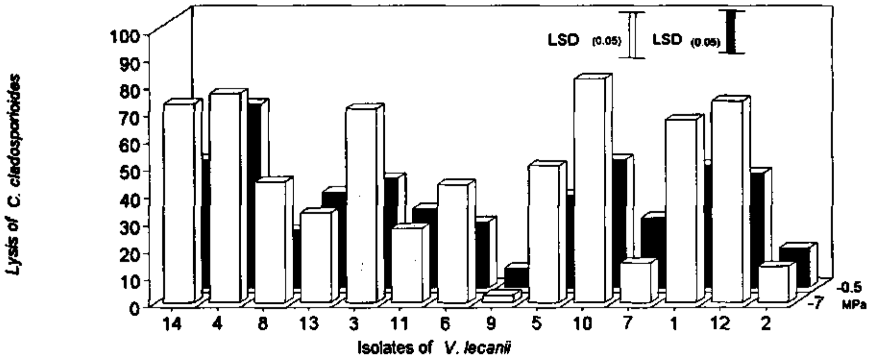


Figure 5. Mean lysis of *C. cladosporioides* parasitized by 14 *V. lecanii* isolates on water agar plates with water potentials of -0.5 and -7MPa. At -0.5 MPa, $LSD_{0.05}$ = 13.3 (n=8) and at -7 MPa, $LSD_{0.05}$ = 16.8 (n=8).

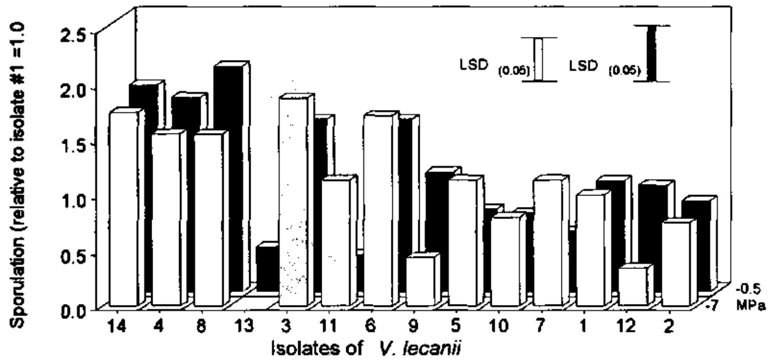


Figure 6. Mean sporulation of 14 *V. lecanii* isolates in relation to isolate 1, growing on water agar plates with water potentials of -0.5 and -7 MPa. At -0.5 MPa, $LSD_{0.05}$ = 0.48 (n=12), and at -7MPa, $LSD_{0.05}$ = 0.39 (n=12).

Table 3. Experiment 7. Combined ANOVA for sporulation of isolates of *V. lecanii* on water agar with water potentials of -0.5MPa or -7MPa.

| Source of variation [a] | Degrees of freedom | Sum of squares | Mean square | F-ratio [b] |
|---|---|---|---|---|
| I | 13 | 119.86 | 9.22 | 38.65 ** |
| H | 1 | 1.43 | 1.43 | 6.00 * |
| R | 2 | 0.04 | 0.02 | 0.08 ns |
| I x H | 13 | 24.55 | 1.89 | 7.91 ** |
| I x R | 26 | 7.13 | 0.27 | 1.15 ns |
| R x H | 2 | 0.23 | 0.12 | 0.50 ns |
| Residual | 22 | 5.25 | 0.23 | |
| Total | 79 | 176.57 | | |

[a] I = isolate, H = humidity, R = replicate experiment (block)
[b] ** significant at $P \leq 0.01$, * significant at $P \leq 0.05$, ns not significant

*Germination of* V. lecanii. Chandler *et al.* (1994) observed the large effect of humidity on conidial germination of *V. lecanii*. They found significant isolate effects and some isolate x humidity interactions, and suggested that quick germination can be of great importance for biocontrol potential. Our data confirmed their findings to some extent.

No correlation was found between germination of isolates of *V. lecanii* on leaves and on agar. Differences between growth and germination on Petri dishes and cucumber leaves suggest that the performance of *V. lecanii* on agar with amended water potentials has little predictive value as to its performance on mildewed leaves with corresponding RH in the ambient air.

*Mycoparasitism on* S. fuliginea. The variation in mycoparasitism on mildew between the two blocks of experiment 2 could be attributed to the level of initial mildew infection. In block I with a high level of mildew, only two isolates of *V. lecanii* (4 and 14) showed adequate mycoparasitism, whereas in block II with less mildew most isolates showed adequate mycoparasitism (Figures 2.1B and 2.2B). The negative correlations between the percentage of mildew parasitized and the number of daughter colonies/dm$^2$ at saturated humidity suggested that reproduction

of mildew is reduced after mycoparasitic attack. After spraying with water, a reduction in number of secondary infections was observed (Figures 2C), possibly because of some antagonism from such saprophytes as *Cladosporium* and *Penicillium* spp (Hijwegen, 1992).

*Mycoparasitism on* C. cladosporioides. The positive correlation between lysis of parasitized *C. cladosporioides* and parasitism on mildew at saturated humidity possibly offers an easy way to screen *V. lecanii* isolates *in vitro* for mycoparasitic ability.

*Isolates*. Isolates 4 and 14 performed well according to most components measured, while isolate 1 (= F88.1 in previous work (Verhaar *et al.*, 1996, Verhaar *et al.*, 1997)) had an intermediate position in the various rankings. Unpublished results indicated that isolate 1 performed better than isolate 4 on cucumber mildew, and that isolates 1 and 13 performed better than isolates 4 and 14 on rose powdery mildew (*Sphaerotheca pannosa* (Wallr.:Fr.) Lév. var. *rosae* Wor.) (Verhaar, unpublished). Isolate 1 has a peculiar history. It was isolated from a strain of *S. fuliginea* and subsequently maintained in the glasshouses of the Laboratory of Phytopathology, Wageningen, for over 20 years. This mildew strain was transferred to fresh cucumber plants every two weeks, about 500 times in 20 years. Assuming that isolate 1 was present all the time, which we cannot possibly prove, and that it coevolved with its mildew host, it could be fairly well adapted to its substrate resulting in a set of unknown specific characters that are necessary for its survival. In our experiments, it never excelled in any of the single characters, yet its overall performance was rather good. *V. lecanii* isolate 2, obtained from the commercial product Mycotal (Koppert BV, Berkel en Rodenrijs, NL), developed in the U.K. for biocontrol of aphids, was less effective against *S. fuliginea* and *C. cladosporioides*. These results suggest host specificity of isolates of *V. lecanii*.

Putative host specificity and other characters not yet tested might have more predictive value than the characters tested so far. For instance, the branching pattern of the mycelium of *V. lecanii* may affect the release of powdery mildew conidia. Mycelium of isolate 1 formed a fine network around powdery mildew hyphae and conidia, with a short distance between the branches in marked contrast to some of the other isolates. pH is another factor possibly causing variation in tritrophic systems which we did not consider. Magan & Lacey (1984) reported that reducing the pH of the substrate from 6.5 to 4 usually increased the relative humidity to allow germination of a group of field and storage fungi, including *V. lecanii*. At 20-25°C, a minimum RH of 90% was necessary for germination at pH 6.5 and 93% at pH 4.0.

*Experimental conditions*. Growth of mildew and *V. lecanii* largely occur in the boundary layer of the cucumber leaves, about 50µm in depth. Little is known about the temperature and humidity at the site of action relative to the temperature and humidity at the site of measurement. Our humidity data must be regarded as rough indications of the humidity in the ambient air, which is good enough for introductory experiments with a miniaturized tritrophic pathosystem.

Experiments with tritrophic systems are complex and vulnerable to disturbances which cannot always be identified and eliminated (Verhaar *et al.*, 1997). The reported experiments with *V. lecanii* on mildewed cucumber leaves showed significant interactions between humidities and blocks. The implications are twofold. First, the technology for tritrophic experiments must be developed further to obtain perfect replications without replicate experiment ('block') effects. Second, the block effects, though undesirable in principle, may provide a valuable insight. Experiment 2, for example, revealed the overwhelming effect of the initial mildew severity on subsequent mycoparasitic events.

*Conclusions and suggestions*. The exploratory study reported here suggests that miniaturized tritrophic experiments are suitable for components analysis of mycoparasitism, when the utmost of care is taken to standardize materials, conditions and actions. Performance of isolates of *V. lecanii* on agar has little predictive value as to their performance on mildewed leaves. However, activity against *C. cladosporioides* on agar plates was a reasonable predictor of activity against powdery mildew on cucumber leaves. Isolates 1, 4, 13 and 14 might be interesting for biocontrol.

# Chapter 6

# Preventative and Curative Applications of *Verticillium lecanii* for Biological Control of Cucumber Powdery Mildew

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Biocontrol Science and Technology 7: 543-551

## Preventative and curative applications of *Verticillium lecanii* for biological control of cucumber powdery mildew

### Abstract

The effect of timing of the application of the mycoparasite, *Verticillium lecanii*, on cucumber powdery mildew, *Sphaerotheca fuliginea*, was studied in a rooted cucumber leaf bioassay. The mycoparasite was applied at different times before and after mildew inoculation. At near-to-maximum humidity (>95% RH), early preventive (9 and 5 days before mildew inoculation) and early curative control treatments (2 days after mildew inoculation) gave considerable reduction in mildewed leaf areas, while late curative treatments resulted in greater mildewed leaf area but ultimately a reduced amount of healthy mildewed leaf area (< 20%). Appropriate timing of biocontrol treatments of *V. lecanii* is important to achieve good control.

### Introduction

In previous experiments, *Verticillium lecanii* (Zimm.) Viégas was shown to be a candidate for biocontrol of powdery mildew (*Sphaerotheca fuliginea* (Schlecht.: Fr.) Poll.) on cucumber (Verhaar *et al.*, 1996). Most research on biocontrol of *S. fuliginea* has concentrated on curative control by mycoparasites (Sundheim, 1982; Philipp and Hellstern, 1986; Jarvis *et al.*, 1989; Bélanger *et al.*, 1994; Urquhart *et al.*, 1994; Verhaar *et al.*, 1996). Few researchers have studied preventative applications (Hoch and Provvidenti, 1979; Knudsen and Skou, 1993). The present work was undertaken to determine the optimum timing for application of *V. lecanii* as a biocontrol agent. Two questions were addressed: (1) What is the most sensitive developmental stage of *S. fuliginea* for biocontrol? (2) Can *V. lecanii* be used preventatively?

## Materials and methods

### Cucumber

Cucumber plants of the susceptible cultivar Corona (De Ruiter Seeds, Bleiswijk, Holland) were grown in a growth chamber (20°C; 16 h light, 23 W/m$^2$ at leaf level, Pope FTL D32W/84HF; 70% relative humidity (RH)). Second and third leaves of three-week-old plants were excised, and each petiole was placed immediately through a hole between two 9 cm plastic Petri dishes, glued together as illustrated by Quinn and Powell (1982). The upper and lower dishes contained the leaf, and 50% Hoagland's solution, respectively. Loss of water from leaves was reduced by closing the double Petri dishes immediately and placing them under shade in the growth chamber for 24 h. Thereafter, Petri dishes were exposed to the light conditions mentioned above for ca. 7 days so that petioles could develop roots in the lower Petri-dish. For each experiment, a homogeneous batch of healthy green leaves was obtained by discarding the most advanced, retarded, and chlorotic leaves.

### Mildew

Rooted leaves mounted as described above were inoculated in a vacuum-operated settling tower (Reifschneider and Boiteux, 1988) using three or four mildewed leaf discs cut with a 1.5 cm cork borer, as the inoculum source. Conidiospores were homogeneously distributed after an interruption of a vacuum of -15 kPa over 24 leaves per treatment. After a settling time of 2 min, leaves were removed from the tower and Petri dishes were closed again. Spore density on inoculated leaves ranged from 130-260 spores cm$^{-2}$. All leaves of each experiment were inoculated in the afternoon of the same day.

### Mycoparasite

*Verticillium lecanii* strain F88.1 was cultured on Oxoid oatmeal agar in Petri dishes at 20°C in the dark. Spores from 8-10-day-old cultures were harvested by washing cultures with demineralised water and filtering the spore suspension through cotton-wool. Spore density was determined with a haemacytometer and adjusted to 5 x 10$^6$ spores ml$^{-1}$. Spore suspensions were applied to the leaves by a de Vilbiss sprayer (van der Kuip Ltd., Utrecht, The Netherlands) until drop formation on the leaf surfaces became visible. Approximately 1.25 ml of spore suspension was applied per leaf resulting in ca. 1.4 x 10$^5$ cm$^{-2}$.

**Biocontrol**

Most experiments compared 8 biocontrol treatments. Each treatment differed in the application date relative to the day of inoculation with mildew (day 0). Preventive treatments were applied on days -9, -5 and -2. On day 0, mycoparasite application preceded mildew inoculation by 4 h to avoid loss of mildew spores by run-off of droplets. Curative treatments were applied on days +2, +5 and +9, when the mildew showed developmental stages of hyphal elongation and haustorium formation (+2), first conidiophores (+5) and abundant sporulation (+9). All treatments were applied at the same time in the afternoon (15.00 h). The control did not receive any treatment.

**Experimental conditions**

All experiments were carried out under optimum humidity conditions, near saturation (>95% RH), for biological control by *V. lecanii* (Drummond *et al.*, 1987). High humidity was obtained by removing lids from upper Petri dishes and placing the mounted leaves on a grid in closed plastic boxes (30 x 45 cm, 30 cm high), with 0.5 l water at the bottom of the box. Twelve mounted leaves fitted into one box and boxes were placed in a growth chamber (20°C, 16 h light, 23 $W/m^2$ at leaf level (Pope FTL D32W/84HF)). To verify humidity and temperature (20°C), small LCD digital thermohygrometers were placed in the boxes (accuracy of ± 1°C and ± 7% RH).

**Experimental design**

Twenty four leaves inoculated simultaneously with mildew formed one block, with eight treatments each of three leaves. One leaf formed one experimental unit. Experiment 1 had four blocks and experiments 2 and 3 each had three blocks. In experiments 2 and 3, mycoparasite spore suspensions were freshly prepared for each block. A randomized complete block design was used. Treatments were placed separately in plastic boxes to avoid cross contamination.

**Assessment**

For each mounted leaf, percentage mildewed leaf area (S) and percentage of the mildewed area affected by the mycoparasite ($S_a$) were assessed. Mildew was considered to be parasitised when the mildew had shrivelled and *V. lecanii* was observed. Visual observations were made using the following scale: 0, 0.1, 0.5, 1, 5, 10, 20,.., 80, 90, 95, 99, 99.5, 99.9 and 100. Percentage leaf area with healthy powdery mildew ($S_h$) was calculated as

$$S_h = S\,(1 - S_a/100).$$

$S_a$ was assessed using a stereo microscope with continuous variable magnification (x 8-50). Assessments were made 5 (experiment 3 only), 7, 9, 12 and 16 days after inoculation with mildew.

**Data Analysis**

Biocontrol effects on total leaf area with powdery mildew and healthy powdery mildew were analyzed using an analysis of variance (ANOVA). The ANOVAs were applied to arcsine square root transformed data to improve homogeneity of error variances (Gomez and Gomez, 1984). The Tukey multiple range test was used for comparison of means. These transversal analyses (Zadoks, 1972) were exemplified by the comparison of means for day 16.

Longitudinal analyses (Zadoks, 1972) were made by comparison of the areas under the progress curve (AUPC) (Campbell and Madden, 1990), mildew progress curve (AUMPC) and healthy mildew progress curve (AUHMPC) of each treatment. The AUMPC and AUHMPC were calculated from day 7 to day 16. The AUMPC of mildew on the control leaves of the experiments was compared by ANOVA and the least significant difference (LSD), taking the control treatments of the three experiments as independent replications. Correlations between experiments for the AUMPC ranking of treatments were assessed by Spearman rank correlation (Hollander and Wolfe, 1973).

During experiments, leaf sizes and leaf conditions were rather uniform. Consequently, no covariates for leaf quality were included in the analysis.

**Additional experiment with half day intervals between curative treatments**

In an additional experiment, six curative treatments on days +2, +4, +4.5, +5, +5.5, and +6 were compared. One preventive treatment was applied on day -9. Controls did not receive any treatment. The experiment had four blocks with eight treatments of three leaves each. Every block was treated with freshly prepared mycoparasite spore suspensions. The experimental conditions were as described above. Observations were made on days 7, 9, 12, 14 and 19. Longitudinal analyses were made by comparison of the areas under the mildew progress curve (AUMPC) and healthy mildew progress curve (AUHMPC) of the treatment, using the Tukey multiple range test.

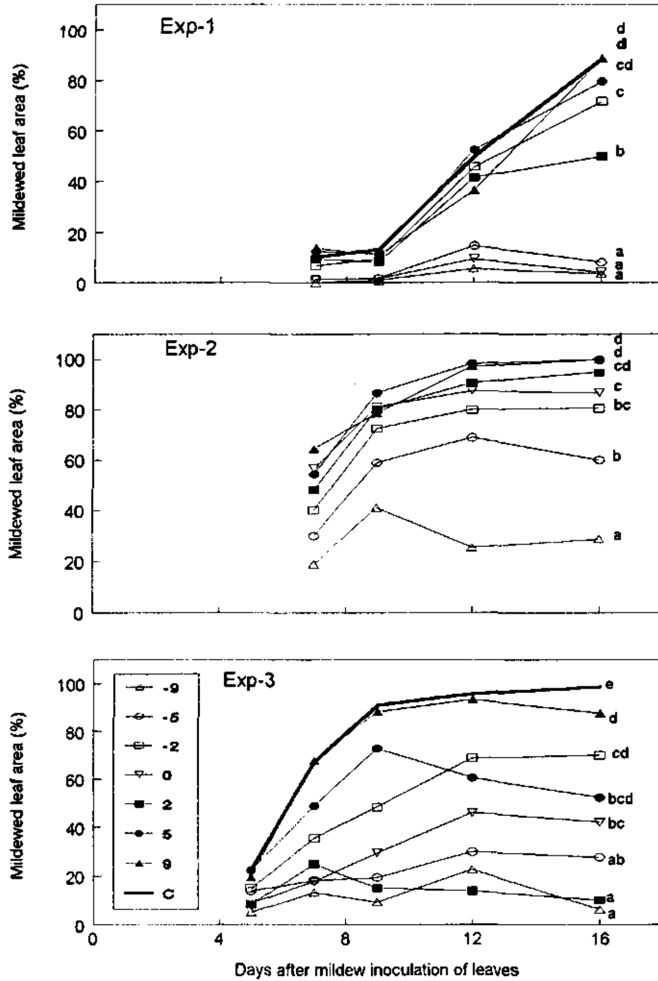


Figure 1. Mean mildewed leaf area after preventive and curative applications of *Verticillium lecanii* in experiments 1, 2 and 3. *V. lecanii* was applied on day -9, -5, -2, 0, +2, +5 and +9 relative to the day of mildew inoculation (day 0). The control did not receive any treatment. Means followed by different letters on the last sampling day (day 16) differ significantly ($P \leq 0.05$) according to Tukey's multiple range test. ($P \leq 0.05$, experiment 1 standard error(SE)=2.3, n=12; experiment 2 SE=3.5, n=9; experiment 3 SE=4.2, n=9).

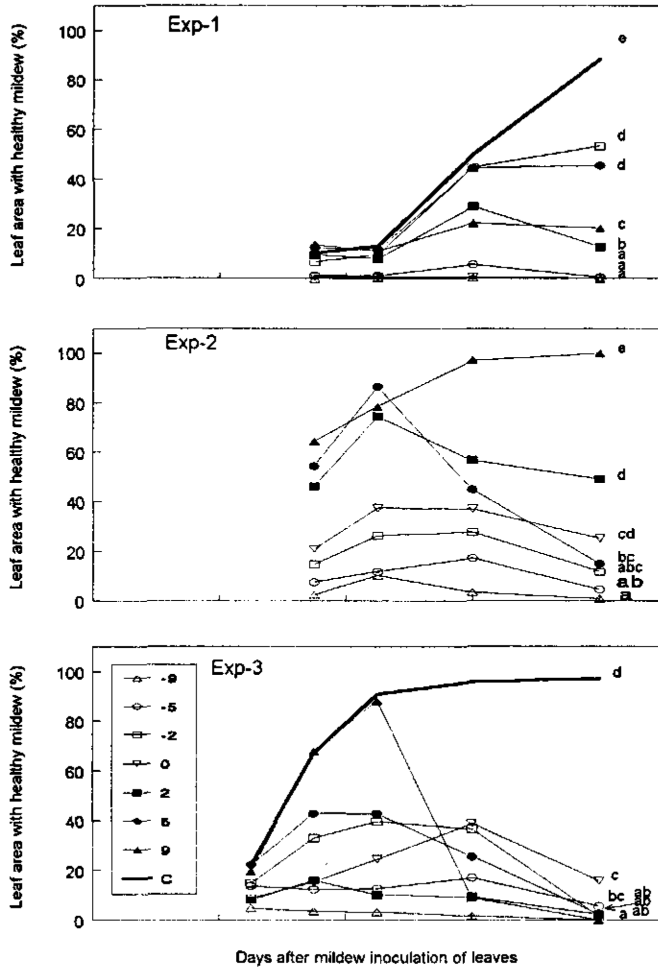


**Figure 2.** Mean leaf area with healthy mildew (not affected by mycoparasites) after preventive and curative applications of *Verticillium lecanii* during experiment 1, 2 and 3. *V. lecanii* was applied on day -9, -5, -2, 0, +2, +5 and +9 relative to the day of mildew inoculation (day 0). The control did not receive any biocontrol treatment. Means followed by different letters on the last sampling day (day 16) differ significantly ($P \leq 0.05$) according to Tukey's multiple range test. ($P \leq 0.05$, experiment 1 SE=1.7, n=12; experiment 2 SE=3.0, n=9; experiment 3 SE=2.7, n=9).

## Results

### Mildewed Leaf Area

The development of mildew in experiments 1, 2 and 3 is shown in Figure 1. Mean percentages of mildewed leaf area were compared for day 16 using the Tukey multiple range test. Results could be classified into one of three groups, somewhat arbitrarily delimited: effective, non-effective and intermediate treatments in comparison with the control.

Effective treatments (code a) were treatment -9 in experiments 1 and 3 (not tested in experiment 2), -5 in all experiments, 0 in experiment 1, and +2 in experiment 3. Non-effective treatments (codes d and e) were the control (C), treatments +9 and +5 in all three experiments, and treatment +2 in experiment 1. All other treatments were intermediate (codes b and c). The results showed some anomalies, such as treatment 0 in experiment 1 and treatment +2 in experiment 3 being comparatively effective. Nevertheless, the trend from relatively high to relatively low control effects corresponded with the trend from early preventive (treatment -9) to late curative (treatment +9) treatments. Mildew development on the control in experiment 2 and 3 was approximately equal. In experiment 1, mildew development was slow, for reasons unknown, but values on day 16 corresponded with those of experiments 2 and 3.

### Leaf Area with Healthy Mildew

Percentages of leaf area with healthy mildew are shown in Figure 2. Mean values for day 16 were compared according to the Tukey multiple range test. Again, treatments could be classified into three groups: effective (leading to low levels of healthy mildew, codes a and b), non-effective (code e in experiments 1 and 2 and code d in experiment 3), and intermediate (code c, and in experiment 1 and 2 code d). Treatments -9 and -5 were effective in all experiments (-9 was not tested in experiment 2). The anomalies were again present; treatment 0 was effective in experiment 1 and treatment +2 in experiment 3.

The most interesting result was shown by treatment +9 in experiments 2 and 3. Mildew developed normally (as control) up to day 9 and then became parasitised so rapidly that on day 16 little healthy mildew was left. In experiment 1, this pattern could not be seen probably because of the retarded mildew development,

but treatment +9 was moderately effective. Treatments +5 and +2 followed the pattern of treatment +9 in all three experiments, but with less extreme peaks and decreases in leaf area with healthy mildew.

In experiments 2 and 3, the trend from relatively high to low effectiveness mentioned above could be seen again with progressively later biocontrol treatments, but an inversion of the trend became visible. This trend inversion was not seen in experiment 1.

### Longitudinal Analysis

Significant treatment effects were found in all experiments (Table 1). In the control, the mildew development in experiment 1 was significantly different from experiment 2 and 3. Spearman Rank correlation coefficients for the area under the mildew progress curve (AUMPC) per treatment (from day 7 to day 16) were calculated for pairs of experiments. Experiments 1 and 3 were positively correlated ($r_s$=0.88, n=8, *P*=0.02), while the other two combinations were positively correlated (both $r_s$=0.71, n=7, *P*=0.08), though not significantly at the 5% level.

**Table 1.** Areas under the mildew progress curve (AUMPC) and under the healthy mildew curve (AUHMPC) of different biocontrol treatments. *V. lecanii* was applied on day -9, -5, -2, 0, +2, +5 and +9 relative to the day of mildew inoculation (day 0).

| | AUMPC | | | AUHMPC | | |
|---|---|---|---|---|---|---|
| Treatments | Exp-1 | Exp-2 | Exp-3 | Exp-1 | Exp-2 | Exp-3 |
| -9 | 29.1 a[a] | | 128.8 a | 2.7 a | | 26.2 a |
| -5 | 72.1 a | 268.9 a | 227.5 ab | 24.8 a | 42.4 a | 159.3 ab |
| -2 | 334.5 bc | 538.3 b | 537.5 c | 293.7 c | 106.7 ab | 274.0 cd |
| 0 | 47.4 a | 664.1 bc | 337.2 b | 5.2 a | 214.0 bc | 243.8 cd |
| +2 | 276.2 b | 739.2 c | 131.9 a | 156.2 b | 295.8 c | 89.2 ab |
| +5 | 384.6 c | 751.6 c | 547.2 c | 288.3 c | 533.0 d | 268.8 cd |
| +9 | 347.5 bc | 792.7 c | 761.7 d | 160.0 b | 454.6 d | 318.3 d |
| Control | 395.9 c (A)[b] | 800.6 c (B) | 827.0 d (B) | 395.9 d (A) | 800.6 e (B) | 821.7 e (B) |
| LSD $P \leq 0.05$ | 78.8 | 151.9 | 148.1 | 64.8 | 122.8 | 124.1 |

[a] Values with different letters are significantly different at $P \leq 0.05$ level according to the Tukey multiple range test.

[b] Values with different capital letters in brackets are significantly different at $P \leq 0.05$ level according to the least significant difference (LSD) test (LSD=64).

Table 2. Additional experiment. Areas under the mildew progress curve (AUMPC) and under the healthy mildew curve (AUHMPC) for eight biocontrol treatments. *V. lecanii* was applied on day -9,2, 4, 4.5, 5, 5.5, and 6 relative to the day of mildew inoculation (day 0). The control received no treatment.

| Treatment | AUMPC | AUHMPC |
|---|---|---|
| -9 | 180.3 abc[a] | 90.7 a |
| 2 | 92.6 a | 37.9 a |
| 4 | 154.5 ab | 76.4 a |
| 4.5 | 110.8 a | 49.3 a |
| 5 | 275.6 bc | 109.3 a |
| 5.5 | 302.7 c | 92.9 a |
| 6 | 472.7 d | 129.2 a |
| Control | 993.3 e | 908.3 b |
| LSD[b] $P \leq 0.05$ | 147.1 | 92.3 |

[a] Values with different letters are significantly different at $P \leq 0.05$ level according the Tukey Multiple Range test.

[b] Values for least significant difference are given.

## Additional Experiment

AUMPCs between day 7 and 19 were compared according to the Tukey multiple range test (Table 2). Again, the visible trend from relatively low to relatively high mildew values corresponded with the trend from early preventive to late curative treatments. AUMPCs showed significant treatment effects. No significant differences were found between AUHMPCs of the various biocontrol treatments.

## Discussion

The experimental conditions, with high humidity, were chosen to optimize the development of the mycoparasite. *V. lecanii* was observed on all treated mildew, whereas the presence of other mycoparasites and of saprophytic fungi was negligible in comparison with the amount of *V. lecanii*. Microscopic observation revealed that *V. lecanii* could even develop on cucumber leaves without mildew infections, presumably feeding on leaf exudates. In principle, free leaf surface water may partially control powdery mildew (Yarwood, 1939), but this was never observed for cucumber powdery mildew (Verhaar, unpublished). In the experiments

presented here, treatment with water only as a control was omitted because it would have favoured interfering saprophytic fungi (Verhaar, unpublished), thereby blurring the findings of the experiments.

Experiments with a tritrophic ecosystem, as reported here, appear to be sensitive to yet unidentified disturbances. In experiment 1, mildew developed late; in experiment 2 the mycoparasite was rather ineffective; in experiment 3 the results were as expected (Figures 1,2). Nevertheless, the constant element in these three experiments was the trend from high to low effectiveness in ranking of the treatments, from early preventative to late curative.

Hoch and Provvidenti (1979) obtained complete prevention of powdery mildew with treatments of *Tilletiopsis* sp. up to 8 days before inoculation with *S. fuliginea*. They did not observe any growth of *Tilletiopsis* sp. in the absence of powdery mildew on cucumber leaves, whereas Knudsen and Skou (1993) found about 90% coverage of cucumber leaves by *T. albescens* at high humidity in the absence of mildew. They tested different application times of *T. albescens* on cucumber mildew at 80-100% RH and found little effect of a preventative treatment with *T. albescens* on day -3. Curative application on the day of inoculation had some effect; treatment on day +3 gave good control whereas treatment on day +7 had moderate effect only. The good control by *Tilletiopsis* sp. applied at day -8 (Hoch and Provvidenti, 1979) and the poor effect of *T. albescens* treatment at day -3 (Knudsen and Skou, 1993) were comparable to our results with *V. lecanii* treatments -9 and -2. We hypothesize that two processes are involved in the biocontrol of powdery mildew by *V. lecanii* and possibly also by *T albescens*: (a) a saprophytic and (b) a mycoparasitic development. For saprophytic fungi, water offers the 'life space' and is the medium of exchange with the leaf and the other members of the phyllosphere community (Ruinen, 1961). Fokkema (1981) stated that most nutrients probably arrive at the plant surface by leaching from within. Guttation will be important for the growth of phyllosphere fungi, especially at high humidity (Frossard, 1981). Water will be drained along the veins, while capillary water can be held by surface structures such as trichomes (Ruinen, 1961).

In our experiments, spores of *V. lecanii* that arrived near leaf veins and trichome bases, probably places with high humidity and good nutrient supply, began to grow rapidly and within one week the fungus developed mycelial networks with good local sporulation. The good control results of the early treatments (-9 and -5) suggest that *V. lecanii*, growing saprophytically, needs at least 5 days to grow to a developmental stage from which it can successfully parasitize young powdery mildew hyphae.

With curative treatments, *V. lecanii* was able to grow in the vicinity of powdery mildew. Only the early treatment (+2) considerably reduced the mildew development on the leaves (experiments 1 and 3). Obviously, the mycoparasite had enough time to germinate and parasitize the young mildew before the mildew sporulated abundantly and covered the leaves. The early (+2) and the late (+9) curative treatments resulted in low levels of healthy mildew, though developmental patterns differed significantly, whereas the intermediate curative treatment (+5) was less effective. Slow mildew development in experiment 1 may have caused the absence of a typical trend inversion of the curative treatments. We hypothesize that *V. lecanii* developed faster on well advanced 9-day-old mildew colonies than on 5-day-old mildew colonies just beginning to sporulate.

In conclusion, timing of biocontrol treatments with *V. lecanii* seems to be very important in achieving successful control. At optimum conditions, early preventive and early curative *V. lecanii* treatments gave the highest reduction in mildew development.

# Chapter 7

# Comparison of three biological control agents against cucumber powdery mildew (*Sphaerotheca fuliginea*) in semi-commercial-scale glasshouse trials

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# Appendix: Detailed observations on the sustainability and development of *Verticillium lecanii* in a glasshouse

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## Comparison of three biological control agents against cucumber powdery mildew (*Sphaerotheca fuliginea*) in semi-commercial-scale glasshouse trials

### Abstract

The effect of three reported biological control agents, *Ampelomyces quisqualis*, *Verticillium lecanii* and *Sporothrix flocculosa*, was tested against cucumber powdery mildew (*Sphaerotheca fuliginea*). Two glasshouse experiments, one in the summer and one in winter/spring were conducted on a semi-commercial scale. In both experiments, a susceptible and a partially resistant cultivar were used. In the second experiment, the additional effect of integration of biological control and silicon amendments to the nutrient solution was also assessed. In both experiments, *A. quisqualis* did not control the disease. *V. lecanii* had a small effect on powdery mildew in the first experiment but not in the second. *S. flocculosa* gave the best control of powdery mildew in both experiments. In the first experiment, weekly application of *S. flocculosa* reduced disease in the partially resistant cultivar to the same level as a treatment in which the fungicides bupirimate and imazalil were each applied once. Addition of silicon in the nutrient solution in a concentration 0.75 mM reduced disease by 10-16%, averaged over all treatments. There was no interaction between silicon and the biocontrol agents. Yield was recorded in the second experiment and was significantly increased by the fungicide treatment compared to the control in the partially resistant cultivar. Yield in the treatment with *S. flocculosa* was not significantly different from the fungicide treatment in this cultivar. Silicon had no effect on yield in either cultivar.

### Introduction

Powdery mildew, caused by *Sphaerotheca fuliginea* (Schlechtend.:Fr.) Polacci, is the most important disease in glasshouse-grown cucumbers in the Netherlands, requiring high inputs of fungicides for control. The intensive use of pesticides is regarded undesirable both for environmental reasons and for the risk of the development of resistance by the pathogen. Furthermore, biological control of insects, which has become common practice, may be adversely affected by powdery mildew fungicides. Therefore, alternative control measures for powdery mildew need to be developed in order to decrease the fungicide input and the dependance on these fungicides.

Biological control of various powdery mildew fungi has been studied quite extensively in the past and was recently reviewed for greenhouse crops (Elad *et al.*, 1996; Menzies and Bélanger, 1996). A few microorganisms have been shown to give moderate to good control under experimental conditions. For instance, *Ampelomyces quisqualis* Ces., a hyperparasite of several powdery mildew fungi, was shown to control *S. fuliginea* in cucumber by a number of researchers (Jarvis and Slingsby, 1977; Philipp and Crüger, 1979; Sundheim, 1982; Sztejnberg *et al.*, 1989). *A. quisqualis* penetrates and feeds on the hyphae of the powdery mildew fungus (Hashioka and Nakai, 1980). A strain of *A. quisqualis*, isolated in the Hebrew University of Jerusalem, Israel (Sztejnberg *et al.*, 1989), has been formulated and commercialized by Ecogen Inc. under the trade name AQ10.

The fungus *Verticillium lecanii* (Zimm.) Viégas is another hyperparasite reported to reduce powdery mildew in cucumber both on leaf disks and in glasshouse experiments (Askary *et al.*, 1997; Spencer and Ebben, 1983; Verhaar *et al.*, 1993; Verhaar *et al.*, 1996). In a comparative study in glasshouse-grown cucumber, *V. lecanii* showed better control of powdery mildew than *Sporothrix rugulosa* (Verhaar *et al.*, 1996).

The yeast-like fungus *Sporothrix flocculosa* Traquair, Shaw and Jarvis (syn. *Pseudozyma flocculosa*) (Boekhout, 1995) was effective against both rose and cucumber powdery mildew (Bélanger *et al.*, 1994; Hajlaoui and Bélanger, 1991; Jarvis *et al.*, 1989). In comparative experiments under controlled conditions, *S. flocculosa* showed more rapid colonization of powdery mildew colonies than *S. rugulosa* or *Tilletiopsis washingtoniensis* and was less affected by unfavourable climatic conditions (Hajlaoui and Bélanger, 1991). The effect of *S. flocculosa* is not based on hyperparasitism, but on antibiosis (Benyagoub *et al.*, 1996; Choudhury *et al.*, 1994; Hajlaoui *et al.*, 1992).

Other fungi, such as *Tilletiopsis* spp. have been reported to control powdery mildews in small scale experiments (Urquhart *et al.*, 1994). However, the performance in glasshouse trials has been disappointing, probably due to low humidity conditions (Hijwegen, 1992).

The efficacy of biocontrol agents depends on the climatic conditions in the crop. Powdery mildew fungi can thrive under dry conditions, whereas most biocontrol agents require relative humidities above at least 70% (Hajlaoui and Bélanger, 1991; Phillip and Helstern, 1986). Furthermore, the rate of development of the powdery mildew may influence the reduction achieved by biocontrol agents, especially in the case of hyperparasites. This means that the efficacy of biocontrol

agents may differ from season to season, from cultivar to cultivar and may be influenced by other control measures.

The objective of this work was to compare the efficacy of *A. quisqualis*, *V. lecanii* and *S. flocculosa* on *S. fuliginea* on glasshouse-grown cucumber under semi-commercial conditions. In order to test the robustness of the biocontrol agents, experiments were run in a summer crop and a winter/spring crop of cucumber, both with a susceptible and a partially resistant cultivar. In the second experiment, the additional effect of integration of biological control and silicon amendments to the nutrient solution was also assessed. The objective was to determine if silicon amendments, reported to reduce the rate of development of powdery mildew (Adatia and Besford, 1986; Menzies *et al.*, 1991; Dik and Voogt, unpublished results) influenced the performance of the biocontrol agents.

## Materials and Methods

*Plant material.* Long English cucumber plants (*Cucumis sativus* L.) of a susceptible cultivar (Jessica in exp. 1, Ventura in exp. 2) and a partially resistant cultivar (Flamingo) were grown in a commercial nursery and transplanted at the four to five leaf stage. The plants were grown in rockwool slabs and trained using the umbrella system (Jarvis, 1992). Fruits were harvested three times per week. Insects were controlled biologically.

*Climate regime.* Heating temperature in the glasshouse was set at 21-22 °C. Ventilation temperature was set at 0.5 °C above the heating point. Screens in the top of the glasshouse were closed from sunset until sunrise, in order to prevent heat loss and to increase relative humidity during the night. Extra $CO_2$ was added to each compartment. Data on temperature, relative humidity and vapour pressure deficit (VPD) were collected in each compartment at one-minute intervals. Averages of 60 minutes were stored in a VAX mainframe computer (Digital, Utrecht, The Netherlands).

*Supply of water, nutrients and silicon.* The plants were grown in rockwool with reuse of drainage water. The nutrient solution was supplied by means of trickle irrigation. The amount was automatically adjusted to the irradiation level. Approximately 30% of the nutrient solution supplied was drained off and reused. The basic composition of the nutrient solution was 1.0 $NH_4$, 6.5 K, 2.75 Ca, 1.0 Mg,

Table 1. Dates of applications of treatments for the two cultivars in both experiments.

| | Experiment 1 (1994) | | Experiment 2 (1995) | |
|---|---|---|---|---|
| treatments | Susceptible cv. | Resistant cv. | Susceptible cv. | Resistant cv. |
| Control[a] | June 14,20,27, July 5 | June 14,20,27, July 5,13,19 | March 3,14,21,28 | March 3,14,21,28, April 5,11,19,26, May 3 |
| *A.quisqualis* | June 14,20,27, July 5 | June 14,20,27, July 5,13,19 | March 3,14,21,28 | March 3,14,21,28, April 5,11,19,26, May 3 |
| *V.lecanii* | June 14,20,27, July 5 | June 14,20,27, July 5,13,19 | March 3,14,21,28 | March 3,14,21,28, April 5,11,19,26, May 3 |
| *S.flocculosa* | June 14,20,27, July 5 | June 14,20,27, July 5,13,19 | March 3,14,21,28 | March 3,14,21,28, April 5,11,19,26, May 3 |
| Fungicide[b] | June 20 (bupirimate), July 13 (imazalil) | June 20 (bupirimate), July 13 (imazalil) | March 17 (bupirimate), March 30 (bitertanol) | March 30 (bitertanol), April 18 (bitertanol) |

[a] in exp. 1: Tween 80 and paraffin oil, in exp. 2: paraffin oil; [b] concentrations of fungicides were 200 ml per 100 l for bupirimate (Nimrod), 100 ml per 100 l for bitertanol (Baycor) and 25 ml per 100 l formazalil (Fungaflor).

11.75 $NO_3$, 1.0 $SO_4$ and 1.25 $H_2PO_4$ in mM and 15 Fe, 10 Mn, 5 Zn, 25 B, 0.75 Cu and 0.75 Mo in $\mu$M. The EC in the root environment was kept between 3.0 and 3.5 $dS.m^{-1}$ and the pH between 5 and 6. In experiment 2, additional Si in a concentration of 0.75 mM was added to half of the plots as potassium metasilicate (9.1% Si, 25.4% K, Sikal, Hydro Agri, Vlaardingen, The Netherlands), for which nitric acid was added in a molar ratio of 2 mol $H^+$ to 1 mol Si to adjust the pH. This resulted in plots with no additional silicon ($Si^-$) and plots with silicon ($Si^+$). The increase in K and N supply by the Si and nitric acid application was equally settled by reduction of the K and N supply by the fertilisers. Drainage water was analysed every two weeks for macro- and micro- elements, Si, EC and pH. If necessary, adjustments to the basic composition of the nutrient solution were carried out.

*Biocontrol agents and preparation of suspensions*. The biocontrol agents tested in both experiments were prepared as follows. *V. lecanii* strain F88.1 was supplied by

Verhaar as a fresh liquid suspension and was diluted to 5 * $10^6$ spores $ml^{-1}$ in 0.3% light white oil (Sigma). *A. quisqualis* was used as the formulated product AQ10, provided by Ecogen Inc. (Langhorne, PA, U.S.A.). It was suspended at a rate of 6 g $l^{-1}$ in 0.05% Tween 80 in exp. 1 and in 0.3% light white oil (Sigma) in exp. 2. *S. flocculosa* was provided by Bélanger as a formulation of dry spores. It was suspended in water with 0.02% Aqua Aid (Ken Crowe Ltd., Montreal, Canada), stirred in a blender and diluted in 0.3% light white oil (Sigma) to a final concentration of ca. 1 * $10^6$ colony forming units (CFU) $ml^{-1}$. The oil, Aqua Aid and Tween 80 amendments were used to enhance survival of the biocontrol agents and to improve homogeneous distribution of the spray solution on the leaves.

*Inoculation with powdery mildew.* Cucumber plants cv. Jessica were grown in a separate small glasshouse compartment and infected with *Sphaerotheca fuliginea*. Spores were blown off the leaves 36 hours before the leaves were used for inoculation of the large glasshouse experiments, in order to ensure that all the spores in the suspension were fresh and of the same age. The source leaves were picked and washed in water. The spore concentration was assessed with a haemocytometer and adjusted to 100 spores $ml^{-1}$. Within two hours of suspending the spores in water, leaf 5 of all the plants in the glasshouse was inoculated with 5-10 ml per leaf. Floors in the glasshouse were wetted and inoculation took place late in the day in order to ensure sufficiently high relative humidity. Inoculation took place 6 days after planting in exp. 1 and 38 days after planting in exp. 2. Inoculation dates were chosen according to the expected first natural infection by powdery mildew in each season.

*Experimental design and treatments.* Both experiments were carried out in a glasshouse with ten compartments of 156 $m^2$ each, five compartments on each side of a corridor. The first experiment was planted on June 2, 1994, and the second experiment on January 17, 1995. One half of each compartment was planted with the susceptible cultivar, the other half with the partially resistant cultivar. Plant density was 192 plants per compartment in exp. 1 and 240 plants per compartment in exp. 2.

In both experiments, five treatments were applied, each replicated in two compartments: 1. control treatment; 2. fungicide according to commercial practice (Table 1); 3. *V. lecanii*; 4. *A. quisqualis*; 5. *S. flocculosa*. In exp. 1, the control consisted of two treatments, each applied to half the plants of each cultivar, i.e. Tween 80 (0.05%) as control for *A. quisqualis* and light white oil (Sigma, 0.3%) as

control for the other biocontrol agents. In exp. 2, the control consisted only of the oil (0.3%), since in this experiment *A. quisqualis* was mixed with paraffin oil instead of Tween 80. All treatments except the fungicide treatment were applied weekly. Spraying dates are shown in Table 1. The treatments were applied with a 10-l knapsack sprayer (Gloria 172RT, Gloria - Werke, Wadersich, Germany) at 3 atm. and a rate of 1500 l $ha^{-1}$ for full-grown plants. A different sprayer was used for each treatment to prevent cross contamination. Applications were done during the last 4 hours before sunset (evening in exp. 1, afternoon in exp. 2) in order to prevent excessive drying of plants after application and subsequent desiccation of biocontrol agents.

Each compartment contained two blocks of six different nutrient solutions, one complete block per cultivar. In exp. 1, all nutrient solutions were the same and the experiment was designed as a randomized complete block with two compartments per treatment as replicates. In exp. 2, silicon in the form of potassium metasilicate was added to three of the six nutrient tanks as described above. The experiment was set-up as a split-plot experiment with two randomized blocks with five treatments and three replicates of two nutrient solutions per compartment per cultivar.

*Disease assessment.* Powdery mildew infection was assessed on 12 plants per compartment per cultivar. Each nutrient tank supplied one plot of four rows of five plants per cultivar. In both experiments, the two middle plants of the six plots per cultivar were used for disease assessments. In exp. 2, six of the 12 plants were from plots with extra silicon and six plants from plots with standard nutrient solution. Infection was assessed as percentage leaf area covered with powdery mildew on all the leaves by position, with leaf 1 as the first full leaf. A set of drawn examples of which the exact percentage infected area was calculated with a semi-automatic image analyser (Videoplan, Carl Zeiss B.V., Weesp, The Netherlands) was used to calibrate assessments. Dead leaves and other diseases were also recorded.

The average percentage dead and diseased leaf area was calculated per plant and added to give the percentage not-green leaf area. Since severe powdery mildew infection can result in death of entire leaves, percentage not-green leaf area gives a better estimate of powdery mildew severity than percentage diseased leaf area. The percentage not-green leaf area was also calculated for three different leaf layers separately, i.e. a base leaf (leaf 6 in both experiments), a leaf in the middle of the canopy (leaf 13 in exp. 1, leaf 10 in exp. 2) and a leaf at the top of the canopy (leaf 20 in exp. 1, leaf 14 in exp. 2), in order to establish the effect of the biocontrol

agents on different parts of the canopy. Data for whole plants and per leaf layer were averaged for the sampled plants per plot.

The first disease assessment was done before the first application of the treatments in order to assess possible differences between compartments. Disease was assessed twice per week in the susceptible cultivar and once per week in the partially resistant cultivar.

*Assessment of silicon in the nutrient solution and in the leaves.* On days 52, 83 and 113 after planting in the fungicide treatment in exp. 2, 10-15 leaves were sampled at different levels in the canopy (base, top and side shoots). The leaves were dried and ground and dry weight was assessed. Silicon was extracted according to Walinga *et al.* (1989) and measured by atomic absorption at 251.6 nm in a nitrous oxide-acetylene flame.

*Populations of biocontrol agents.* In both experiments, populations of the biocontrol agents were assessed at different times after spraying, on different leaf layers and cultivars to determine survival on the leaves. For *V. lecanii*, 12 leaf disks per cultivar per replicate were sampled at two heights in the canopy and incubated at high humidity. Subsequent growth of the fungus was observed using a stereo microscope. In exp. 2, germination of *V. lecanii* spores both before and after incubation at high humidity, was assessed microscopically for 100 spores per leaf disk.

On several sampling dates in exp. 1, leaf disks were examined microscopically for the presence of *A. quisqualis* and parasitization of the powdery mildew. Colonization of powdery mildew by *A. quisqualis* was assessed on whole plants on one sampling date in exp. 2 as percentage leaves on which powdery mildew was visibly colonized by the hyperparasites in three classes: no parasitization, < 50% of powdery mildew parasitized, and > 50% parasitized.

Population densities for all three biocontrol agents were assessed by sampling leaf disks at different heights in the canopy, washing the samples in sterile Tween 80 (0.01%) and dilution plating on PDA plates. Plates were counted after incubation at 21 °C for 2-8 days and the population density, expressed as CFU $cm^{-2}$, was calculated. Samples were taken both in the compartments where the biocontrol agents were sprayed and in the control compartments to assess possible cross-contamination.

*Yield.* The number, weight and quality of the harvested fruits were recorded. Quality was recorded as first or second class, depending on size, colour and shape of the fruits using the same criteria as the auction. Total weight and number of fruits per plant and the percentage fruits of first class quality were calculated at the end of the experiments. In exp. 1, yield was assessed for one row of 12 plants per cultivar per compartment. In exp. 2, yield was assessed for 5 plants for each of the six nutrient plots for each cultivar in each compartment.

*Statistical analysis.* The Area Under the Disease Progress Curve (AUDPC) for percentage not-green leaf area was calculated, both for whole plants and for the three different leaf layers. Exp. 1 was analysed as a complete block, exp. 2 as a split-plot experiment with the treatments as main factor and Si level in the nutrient solution as factor within the treatments. AUDPC values and yield data were subjected to analysis of variance followed by Fisher's protected LSD test, at $P=0.05$.

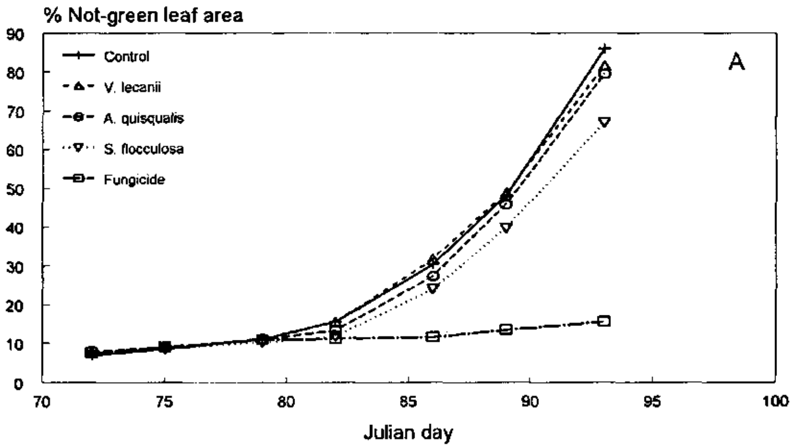
Percentage inhibition in AUDPC compared to the control treatment was calculated both for whole plants and for the three separate leaf layers. Linear regression analysis was performed on the percentage inhibition in AUDPC against leaf layer for each biocontrol agent and cultivar combination. Differences in elevations and slopes of the regression lines were analysed according to Snedecor and Cochran (1980) at $P=0.05$. All statistical analyses were done with Genstat (Genstat 5 Committee, 1992).

## Results

*Climatic conditions in the glasshouse.* In the summer of 1994 (exp. 1), conditions in the glasshouse were warm and dry. The 12 h average temperatures ranged between 19 and 32 °C and 12 h average relative humidity (R.H.) was between 30 and 80%, resulting in VPD's between 0.4 and 3.0 kPa.

In exp. 2 (spring 1995) 12 h average temperatures ranged between 19 and 26 °C. The 12 h average R.H. was between 55 and 90%, resulting in VPD's between 0.2 and 1.5 kPa.

In both experiments, differences between compartments were within 0.3 °C and 0.1 kPa.



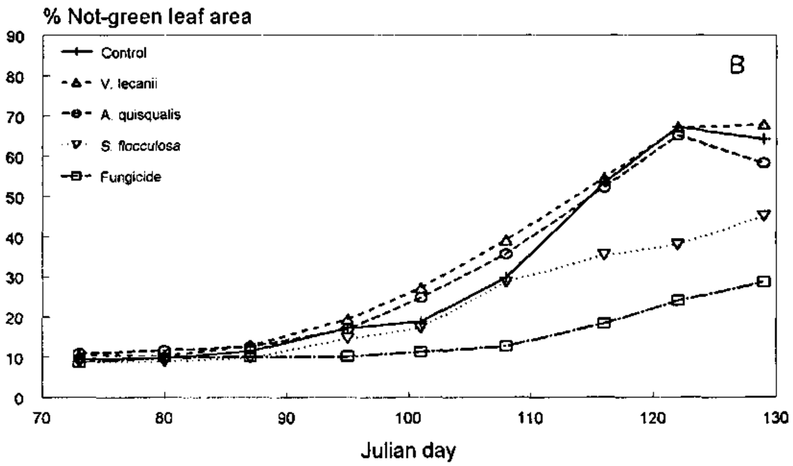




Fig. 1. Disease progress of powdery mildew on cucumber in exp. 2 for all treatments without silicon in the nutrient solution for the susceptible cultivar Ventura (A) and the partially resistant cultivar Flamingo (B)

*Powdery mildew epidemics.* Inoculation of leaf 5 resulted in a few powdery mildew colonies on this leaf within one week in both cultivars in exp. 1 and the susceptible cultivar in exp. 2 and within two weeks in the partially resistant cultivar in exp. 2. Before the first application of the biocontrol agents, no differences were observed among treatments in both experiments. In both seasons, the experiments were terminated earlier for the susceptible cultivar than for the partially resistant one by spraying the susceptible plants in all treatments with a fungicide (imazalil in exp. 1, bupirimate in exp. 2) when disease became very severe. Since the plants of both cultivars were grown in the same compartments, the susceptible plants were providing an unnaturally high inoculum level for the partially resistant plants. This application of chemical fungicide to all susceptible plants was done on July 13 in exp. 1 and on April 4 in exp. 2, 35 and 39 days after inoculation with powdery mildew, respectively.

*The effect of the biocontrol agents and silicon on powdery mildew and other diseases.* In general, the development of the epidemic was delayed by *S. flocculosa* in both experiments and by *V. lecanii* in exp. 1. This was especially noticeable in the partially resistant cultivar. Eventually however, disease reached a very high level in all treatments except the fungicide treatment. Disease progress in all treatments without silicon in exp. 2 is shown in Fig. 1. In exp. 1, there was a distinct inhibitory effect of Tween 80 on powdery mildew development in both cultivars compared to paraffin oil. Compared to Tween 80, *A. quisqualis* did not reduce powdery mildew severity. In exp. 2, *A. quisqualis* mixed with paraffin oil instead of Tween 80, had no effect on powdery mildew in either cultivar.

The Area Under the Disease Progress Curve (AUDPC) for not-green leaf area is given for both experiments in Tables 2 and 3. *V. lecanii* and *A. quisqualis* did not cause a significant reduction in the AUDPC in either experiments compared to the appropriate control. Only *S. flocculosa* significantly reduced the AUDPC. For the partially resistant cultivar, the AUDPC in the *S. flocculosa* treatment was not significantly different from the fungicide treatment in exp. 1.

Table 2. AUDPC for powdery mildew, expressed as percentage not-green leaf area in the susceptible cultivar.

| Treatment | AUDPC [a] (% - days) | | |
|---|---|---|---|
| | Exp. 1[b] | Exp. 2[b] | |
| | | - Si | + Si |
| Control Tween | 289 | * | * |
| Control paraffin oil | 425 | 582 | 468 |
| *A. quisqualis* | 300 | 582 | 521 |
| *V. lecanii* | 348 | 545 | 496 |
| *S. flocculosa* | 306 | 477 | 383 |
| Fungicide | 141 | 241 | 184 |
| LSD[c] | 114 | 64 | 64 |

[a] AUDPC = Area Under the Disease Progress Curve. [b] The total number of days in the disease assessment period was 27 in exp. 1 and 21 in exp. 2. [c] LSD = Least Significant Difference at P=0.05.

Table 3. AUDPC for powdery mildew expressed as percentage not-green leaf area in the partially resistant cultivar.

| treatment | AUDPC [a] (% - days) | | |
|---|---|---|---|
| | Exp. 1[b] | Exp. 2[b] | |
| | | - Si | + Si |
| Control Tween | 1206 | * | * |
| Control paraffin oil | 1517 | 1688 | 1572 |
| *A. quisqualis* | 1071 | 1753 | 1638 |
| *V. lecanii* | 1072 | 1618 | 1481 |
| *S. flocculosa* | 787 | 1226 | 1204 |
| Fungicide | 522 | 742 | 685 |
| LSD[c] | 503 | 221 | 221 |

[a] AUDPC = Area Under the Disease Progress Curve. [b] The total number of days in the disease assessment period was 45 in exp. 1 and 56 in exp. 2. [c] LSD = Least Significant Difference at P=0.05.

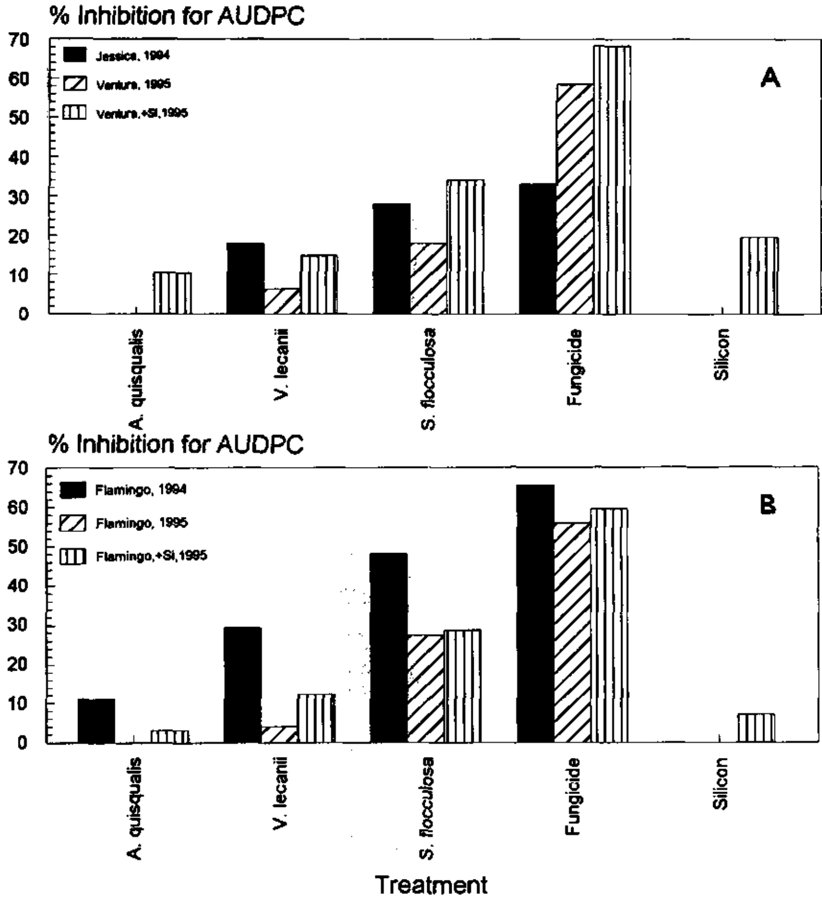



Fig. 2. Percentage inhibition in the Area Under the Disease Progress Curve (AUDPC) of cucumber powdery mildew compared to the control treatments in the susceptible cultivars (A) and the partially resistant cultivar (B) in two experiments.

The effect of silicon in exp. 2 was significant but not very strong (Fig. 2). On average for all treatments, addition of silicon gave 16 and 11 % reduction of powdery mildew for the susceptible and the partially resistant cultivars, respectively. There was no interaction with the biocontrol agents, indicating that reduction of powdery mildew by biocontrol and silicon act independently and the biocontrol agents are not influenced by the silicon in the leaves.

The percentage inhibition obtained by the treatments compared to the control is shown for both cultivars for 1994 and 1995 without silicon and 1995 with silicon in Fig. 2. The effect of all three biocontrol agents was stronger on the partially resistant cultivar than on the susceptible cultivar, especially in exp. 1. For most cultivar/treatment combinations, the effect without silicon was stronger in 1994 than in 1995. In 1995, silicon increased inhibition for all treatments.

In exp. 1, the inhibition of powdery mildew was clearly correlated to the position of the leaves. For *V. lecanii* and *S. flocculosa*, the percentage inhibition in exp. 1 compared to the control is plotted against leaf layer in Fig. 3. *A. quisqualis* showed no control in any of the leaf layers analysed. Better control was achieved by *V. lecanii* and *S. flocculosa* on the lower leaves than on the top leaves. For both these biocontrol agents, the slopes of the regression lines were significantly different from horizontal. No significant differences between slopes of regression lines occurred among the cultivar- treatment combinations. In exp. 2, only *S. flocculosa* showed an effect on powdery mildew. The inhibition achieved in this experiment was not significantly influenced by leaf layer, indicating that the climatic conditions in exp. 2 were not negatively influencing the performance of *S. flocculosa* on the upper leaves.

In exp. 1, infection by *Pythium aphanidermatum* occurred early in the experiment. No effect of any of the treatments was observed. In exp. 2, *Botrytis cinerea* stem infection began to appear in April. At the end of the experiment, the number of dead plants was not influenced by any of the treatments.

*Silicon levels in nutrient solution and leaves.* In exp. 2, the amount of silicon in the recirculating drain solution in the tanks with additional silicon decreased steadily from 0.80 mM in January to 0.44 mM in April. In the control tanks, silicon levels ranged throughout the experiment between 0.09 and 0.12 mM. Differences between tanks with the same silicon treatment were negligible.

The amount of silicon in the leaves of both cultivars is shown for different leaf positions and several sampling dates in Table 4. Silicon levels clearly increased

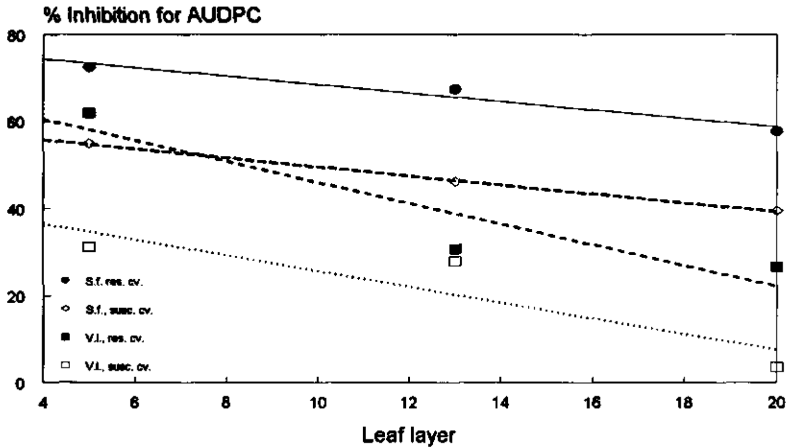



Fig. 3. Percentage inhibition in the Area Under the Disease Progress Curve (AUDPC) of cucumber powdery mildew by *S. flocculosa* and *V. lecanii* compared to the control treatments plotted against leaf layer in exp. 1. V.l. = *Verticillium lecanii*, S.f. = *Sporothrix flocculosa*. Leaf layer 1 is the first full leaf. Equations for regression lines were (Y = percentage inhibition): Y = 46.4 - 1.97 * leaf layer ($R^2$ = 0.84) for *V. lecanii* in the susceptible cultivar; Y = 72.5 - 2.53 * leaf layer ($R^2$ = 0.84) for *V. lecanii* in the partially resistant cultivar; Y = 61.1 - 1.1 * leaf layer ($R^2$ = 1.00) for *S. flocculosa* in the susceptible cultivar; Y = 79.6 - 1.1 * leaf layer ($R^2$ = 0.97) for *S. flocculosa* in the partially resistant cultivar.

with time, resulting in substantial differences in silicon content of the leaves between $Si^+$ and $Si^-$ plants. There were no differences in silicon uptake between the two cultivars.

*Population dynamics of the biocontrol agents.* All three biocontrol agents were only found in the compartments in which they had been applied. *V. lecanii* was present on more than 90% of the samples up to one week after spraying in both experiments. At the end of exp. 1, population densities of *V. lecanii* were up to 4 times higher on leaf disks in the middle of the canopy than on the top leaves. The germination rate of *V. lecanii* on sampled leaf disks as assessed in exp. 2 was around 7% on fresh samples and around 38% after incubation under humid

Table 4. Silicon levels in leaves (mmol/kg dry matter) of cucumber plants fed with or without potassium silicate in the fungicide treatment in exp. 2.

| position of leaf | cultivar | March 10 | | April 10 | | May 5 | |
|---|---|---|---|---|---|---|---|
| | | + Si | - Si | + Si | - Si | + Si | - Si |
| base | Flamingo | 658 | 148 | n.d. | n.d. | n.d. | n.d. |
| | Ventura | 671 | 133 | n.d. | n.d. | n.d. | n.d. |
| top | Flamingo | 425 | 104 | 714 | 170 | 864 | 173 |
| | Ventura | 424 | 108 | 705 | 175 | 859 | 199 |
| side-shoot | Flamingo | n.d.[a] | n.d. | 620 | 151 | 738 | 146 |
| | Ventura | n.d. | n.d. | 602 | 164 | 730 | 166 |

[a] n.d.=not determined.

Samples of 10-15 leaves were taken per leaf layer in plots with and without silicon amendments to the nutrient solution.

conditions and 20 °C for 24 hours. Germination was not influenced by leaf layer, cultivar or silicon treatment.

*A. quisqualis* was present on most of the samples in exp. 1 and mostly on samples in the middle of the canopy in exp. 2. Dilution plating showed, that population densities in exp. 1 were quite stable up to one week after spraying.

However, in most of the samples observed microscopically, spores of *A. quisqualis* were found but no parasitism was observed on the leaf disks. In exp. 2, assessment of the parasitism of powdery mildew by *A. quisqualis* on all the leaves of selected plants showed that on 69% of the leaves, powdery mildew was not parasitized, on 23 % of the leaves, less than 50% of the powdery mildew colonies and on 8 % of the leaves, more than 50% of the powdery mildew colonies were parasitized. No differences between the two compartments were observed.

*S. flocculosa* was recovered from all samples. There was no consistent effect of cultivar, leaf layer or silicon treatment (in exp. 2) on population density. However, the population density of *S. flocculosa* was always higher on leaves with powdery mildew infection than on disease-free leaves.

*Yield.* In exp. 2, total yields in the susceptible cultivar were not significantly influenced by any of the biocontrol agents or the silicon amendment and ranged from 4.8 to 5.5 kg plant$^{-1}$. In the partially resistant cultivar, only the fungicide

treatment significantly increased total yields compared to the control. However, in the treatment with *S. flocculosa*, yields were not significantly different from the fungicide treatment. Silicon had no effect on yield.

For both cultivars, the percentage of first class quality fruit was not influenced significantly by the biocontrol agents, the fungicide or silicon and ranged between 74 and 81 %.

## Discussion

To our knowledge, our experiments are the first to compare several biocontrol agents of cucumber powdery mildew in semi-commercial scale experiments. Based on the experimental design which prevented cross contamination and the similar conditions in all compartments of the experiments in terms of nutrition, climate and initial disease pressure, we were able to obtain an unbiased and reliable assessment of the relative efficacy of the biocontrol agents tested.

In both experiments, *A. quisqualis* did not control powdery mildew compared to the controls. *V. lecanii* gave some control in exp. 1 but not in exp. 2. In both experiments, *S. flocculosa* gave the best control of the biocontrol agents, even to the point where the effect was not significantly different from the fungicide treatment. Some of our results contrast with previous reports of efficacy obtained in small-scale experiments. For example, with *A. quisqualis* Jarvis and Slingsby (1977) and Sztejnberg *et al.* (1989) obtained approximately 50% reduction in severity of powdery mildew and an increase in yield in glasshouse-grown cucumber compared to the control. Verhaar *et al.* (1996) found that *V. lecanii* could maintain powdery mildew on an artificially inoculated cucumber crop below 15 % infected leaf area for 9 weeks on the partially resistant cultivar Flamingo. The AUDPC was not significantly different from the fungicide treatment in their first experiment. However, in the replication of the experiment, *V. lecanii* showed no effect on powdery mildew compared to a control treatment with water. The discrepancy between results reported in the literature and our results may be attributed in part to the drier conditions that prevailed in our experiments. In this context, *S. flocculosa* seemed to be the least affected by dry conditions, which is in accordance with findings of Hajlaoui and Bélanger (1991). The climatic conditions during daytime in exp. 1 were quite severe for the biocontrol agents. Irradiation, temperature and vapour pressure deficit were very high and this probably caused the leaf layer effect on performance of both *V. lecanii* and *S. flocculosa*. During the more moderate

conditions in exp. 2 this effect of leaf layer was not as clear. Nonetheless, control in the first experiment was at least as good as in the second experiment. This indicates that the biocontrol agents can survive periods of unfavourable conditions, provided that these periods are alternated with periods of more moderate temperatures and higher humidities, as generally occur during the night. The population studies confirm that all three biocontrol agents survived on the leaves in both experiments. However, activity of *A. quisqualis* and *V. lecanii* was very limited.

Apart from a better tolerance to dry conditions, the more consistent performance of *S. flocculosa* compared to the two other agents may also be attributed to the mode of action of the biocontrol agents. Both *A. quisqualis* and *V. lecanii* are hyperparasites (Askary *et al.*, 1997; Sundheim and Tronsmo, 1988; Yarwood, 1932) and their growth needs to be as fast as that of the pathogen in order to give sufficient control. It was our distinct impression that the powdery mildew colonies in our experiments developed faster than the hyperparasites. Parasitism was observed in the centre of the colonies, but at the edges the pathogen was growing away from the hyperparasites. On the other hand, *S. flocculosa* is not a hyperparasite, but a fungus which excretes antibiotics (Benyagoub *et al.*, 1996; Choudhury *et al.*, 1994; Hajlaoui *et al.*, 1992). The advantage is that the biocontrol agent does not have to be in direct contact with the pathogen, because the molecules will diffuse over the leaf surface.

Additives are commonly used with biocontrol agents to promote survival and to ensure a homogeneous coverage on the plant surface. Glycerol, Tween and different kinds of oil have been shown to improve the performance of biocontrol agents (Hijwegen, 1992; Philipp and Hellstern, 1986; Philipp *et al.*, 1990; Spencer and Ebben, 1983; Verhaar *et al.*, 1996). However, these additives may directly affect powdery mildew (Hijwegen, 1992; Verhaar *et al.*, 1996). In our first experiment, the treatment with only Tween 80 gave significant control of powdery mildew compared to paraffin oil. Since *A. quisqualis* was applied together with Tween 80 and gave similar control as Tween 80 by itself, the relative control of this treatment in this experiment was attributable to the additive only. This is confirmed by the lack of control by *A. quisqualis* in the second experiment, in which it was applied in an oil mixture. We have tested the oil that we used for its effect against powdery mildew in several smaller scale experiments. The results showed, that this oil in all concentrations tested (up to 5%) did not control powdery mildew in cucumber (Dik and Bélanger, unpublished results). Bélanger *et al.* (1994) found that *S. flocculosa* mixed with 1% paraffin oil controlled rose powdery mildew slightly better than the biocontrol agent alone. They ascribed this result to increased survival

of the biocontrol agent rather than to a direct effect of the oil on powdery mildew. The use of additives as controls in our experiments has ensured that we can separate the effect of the biocontrol agents themselves from that of the additives.

In both experiments, control was generally better in the partially resistant cultivar than in the susceptible cultivar. This integration of cultivar and biocontrol should therefore be considered in a general management scheme of powdery mildew. Also, silicon amendments to the nutrient solution provided some additional reduction in powdery mildew severity. Biocontrol may also be integrated with chemical control. The performance of *A. quisqualis* was much better when used in combination with low level fungicide applications (Sundheim, 1982). For *A. quisqualis*, the compatibility with fungicides has been assessed by Philipp *et al.* (1982, 1984) and Sztejnberg *et al.* (1989). For *S. flocculosa* and *V. lecanii*, more information on compatibility with fungicides is needed. In a very susceptible cultivar, integration with chemical fungicides may prove to be necessary.

In spite of their inefficacy in the experiments reported here, both *A. quisqualis* and *V. lecanii* were present on the leaves and started to grow immediately after placing sampled leaf disks under humid conditions. This confirms that the humidity conditions prevailing in the glasshouses limited their growth. No differences occurred in the presence of the biocontrol agents in treatments with different silicon levels, confirming that the silicon and biocontrol agents acted independently. The applied biocontrol agents were only found in the compartments where they were sprayed, so no cross-contamination occurred between compartments. It has been suggested that biocontrol agents may spread with the powdery mildew spores and therefore results may be difficult to interpret (Philipp *et al.*, 1984); in our experiments the separate compartments apparently formed a sufficient barrier.

In general, it can be concluded that of the three biocontrol agents tested, *S. flocculosa* shows the best potential for efficient biocontrol of cucumber powdery mildew under the conditions prevailing in Dutch glasshouses. The experiments were run in the two seasons in which powdery mildew is most severe and under conditions that were similar in all aspects to commercial glasshouse conditions. The control in our experiments was not sufficient for commercial growers, with the exception of the partially resistant cultivar in exp. 1, but in this respect it must be noted that our experiments aimed at testing the performance of the biocontrol agents under severe infection pressure. Artificial inoculation of all plants provided a homogeneous, but at the same time unnaturally high disease pressure quite early in the growing season. The fact that even in this situation significant control occurred

allows optimism with respect to the possibilities of biocontrol under more moderate infection pressures. Further experiments will be needed to provide more information on this aspect. Furthermore, integration with yet other methods, for example induced resistance by means of plant extracts and with chemical control will be the topic of future research.

## Detailed observations on the sustainability and development of *Verticillium lecanii* in a glasshouse

### Introduction

In the foregoing chapter three biocontrol agents of cucumber powdery mildew, *Verticillium lecanii*, *Sporothrix flocculosa* and *Ampelomyces quisqualis* were compared in glasshouse experiments, at the Research Station for Glasshouse Vegetables in Naaldwijk. In contrast to preliminary experiments (Verhaar *et al.*, 1997) *V. lecanii* showed unexpectedly poor control results. Some detailed observations on the fate of *V. lecanii* were made which might explain the failure of *V. lecanii* to control cucumber powdery mildew. The development of *V. lecanii* in the glasshouse was studied by visual observation using a hand lense (x 25) and by sampling. The effect of glasshouse compartment, leaf level and cucumber cultivar on the presence of vital *V. lecanii* propagules, the number of cfu's and the percentage of germinated *V. lecanii* spores on leaf disks was determined. Observations on percentage germination were made on leaf disks sampled 12 hours or one week after biocontrol treatments, before and after incubation at high humidity. Some of these observations were already briefly described in the foregoing chapter.

### Materials and Methods

*Observations during glasshouse experiment 1, summer 1994.*

*The presence of vital* V. lecanii. To study the presence of vital *V. lecanii* the partially resistant cultivar Flamingo was sampled one week after the second (day 181), fourth (day 195) and sixth (day 208) biocontrol treatment. The susceptible cultivar Jessica was sampled one week after the second (day 181), 5 days after the fourth biocontrol treatment (day 193), two days (day 195) and two weeks (day 208) after the fungicide treatment. Plants in the treatment compartments 5 and 10 and in the control compartments 2 and 7 were sampled by taking 12 leaf disks of 3.14 $cm^2$ from the top and middle leaf layers (leaves 13 and 20 approximately). The leaf disks were incubated in plastic Petri dishes (5 cm diameter), on wet filter paper, in a growth chamber (20°C; 16 h light, 23 $W/m^2$, Pope FTL D32W/84HF). After three days the leaf disks were examined for the presence of *V. lecanii* under a stereomicroscope with continuously variable magnification (8 to 50x).

*Number of cfu's.* The number of *V. lecanii* cfu's removed from leaf disks by washing was determined per leaf level of plants with and without *V. lecanii* treatments. Four samples of three leaf disks were taken per combination of cultivar, leaf level and treatment (*V. lecanii* and control). The three leaf disks per sample were taken together and were washed in 100 ml distilled water. After one min shaking on the vibrofix, 0.2 ml (day 193) or twice 0.1 ml (day 208) per sample was plated on water agar (Oxoid) to which chloramphenicol (50 ppm) and streptomycin (50 ppm) had been added. Three days later the *V. lecanii* colonies per Petri dish were counted and the numbers of cfu's per $cm^2$ of leaf disk were calculated.

*Observations during glasshouse experiment 2, spring 1995.*
*The presence of vital* V. lecanii. One week after the second treatment (day 80), 12 hr after the third (day 81) and one week after the fourth and last biocontrol treatment on cv. Ventura (day 101) and 12 hr after the fifth biocontrol treatment on cv. Flamingo (day 102) cucumber plants in the treatment compartments (4 and 7) and in the control compartments (3 and 10) were sampled for *V. lecanii* as described for experiment 1.

*Germination percentage of* V. lecanii. In addition, on each assessment day, 12 leaf disks were sampled per combination of cultivar and leaf level to determine the germination percentage of *V. lecanii*. The samples were transported to Wageningen in a cool box. Six leaf disks per combination were placed in a refrigerator whilst the other six were incubated at high humidity conditions (near saturated atmosphere) for 24 hours at 20°C, d/n 16/8, and 32.2 $W/m^2$ light intensity. *V. lecanii* spores on the leaf disks were coloured by 0.1% fluorescent brightener 28 (Sigma) and viewed under a Zeiss Axioskop equipped with an incident-light fluorescence illumination, a 395-440 nm excitation filter, a 460 nm dichroic mirror and a 470 nm barrier filter. Per leaf disk about 100 spores were examined. A spore was counted as germinated when the length of the germ tube was longer than the length of the spore.

Statistics

*The presence of vital* V. lecanii. The chi-square test for a fixed-ratio (1:1) hypothesis was used to examine whether treatment, cultivar or leaf level had an effect on the presence of viable *V. lecanii*.

*Number of cfu's.* The effects of leaf level and glasshouse compartment on numbers of cfu's were analyzed by ANOVA followed by a least significant difference (LSD) test.

*Germination percentage of* V. lecanii. The effects of leaf level and glasshouse compartment on percentage of *V. lecanii* spores before and after incubation at high humidity were analyzed by ANOVA followed by a least significant difference (LSD) test. To improve homogeneity the data were arcsine square root transformed.

**Results**

*Glasshouse experiment 1, summer 1994*

*Presence of vital* V. lecanii. In the control compartments (2 and 7) no *V. lecanii* was found. In the treatment compartments (5 and 10), *V. lecanii* was found on over 90% of all leaf disks, with one exception. Two days after the fungicide (imazalil) treatment (day 195), the cv. Jessica had 36% of the leaf disks with *V. lecanii* against 98% on cv. Flamingo, a significant difference ($P \leq 0.0001$).

*Number of cfu's.* Five days after the fourth biocontrol treatment the number of cfu's from cv. Jessica (day 193) differed significantly (Table 1, $P \leq 0.05$) between compartments 5 (average $14 \times 10^3$ per $cm^2$) and 10 (average $3 \times 10^3$ per $cm^2$). On day 208, the difference was not significant but indicative ($P \leq 0.1$), one week after the last biocontrol treatment on cv. Flamingo (Table 1).

Taking compartments 5 and 10 together for day 208, the middle leaf layer of cv. Flamingo yielded significantly more cfu's (about 4x, $P \leq 0.05$) than the top leaf layer. Two weeks after the fungicide treatments on cv. Jessica (day 208), cv. Jessica yielded significantly more ($P \leq 0.05$) cfu's than cv. Flamingo plants which were treated with *V. lecanii* one week before (Table 1).

Glasshouse experiment 2, spring 1995

*Presence of vital* V. lecanii. In the control compartments (3 and 10) no *V. lecanii* was found. In the treatment compartments (4 and 7) *V. lecanii* was observed on all sampled leaf disks. Even one week after a fungicide (bupirimate) treatment on cv. Ventura all leaf disks showed vital *V. lecanii.*

*Germination of* V. lecanii *spores.* A significant ($P \leq 0.05$) difference in germination data was found between compartments 7 (11%) and 4 (5%). The

Table 1. Glasshouse experiment 1, number of cfu's per $cm^2$ leaf disk

| Julian day | Compart-ment | Middle leaf layer | | Top leaf layer | |
|---|---|---|---|---|---|
| | | Jessica | Flamingo | Jessica | Flamingo |
| 193 | 5 | 12.4 b[a] | --[c] | 15.7 b | -- |
| | 10 | 3.1 a | -- | 2.9 a | -- |
| 208 | 5 | -- | 4.8 A[b] | 9.7 B | 1.5 A |
| | 10 | -- | 2.9 A | 4.4 B | 0.5 A |

[a] Number of cfu's in thousands per $cm^2$ leaf disk, values in the same column with different letters (lower case) are significantly different at $P \leq 0.05$ level according to the LSD multiple range test.
[b] Values in the same row with different letters (upper case) are significantly different at $P \leq 0.05$ level according to the LSD multiple range test.
[c] Numbers not determined.

period between the last biocontrol treatment, 12 hours or 7 days seemed to have no influence on the percentage germinated spores. However, when part of these samples were incubated at high humidity for 24 hours significantly ($P \leq 0.05$) more spores had germinated on cv. Ventura leaf disks sampled 12 hours after the third biocontrol treatment (day 81, 56%) than on those taken 7 days after the second treatment (day 80, 29%) (Figure 1). On cv. Flamingo no difference in percentage germination was observed on leaves taken 12 hours after the third biocontrol treatment (day 81, 33%) or 7 days after the second biocontrol treatment (day 80, 33%).

On day 101, cv. Ventura had so much powdery mildew that *V. lecanii* spores could no longer be distinguished on the leaf disks. On cv. Flamingo, the percentage germinated spores after treatments 5 and 6 (day 101, 102) was significantly ($P \leq 0.05$) higher than after treatments 3 and 4. No significant differences ($P \leq 0.05$) were found for percentage germinated spores between compartment, leaf level and time after the last treatment, before and after incubation (Figure 1).

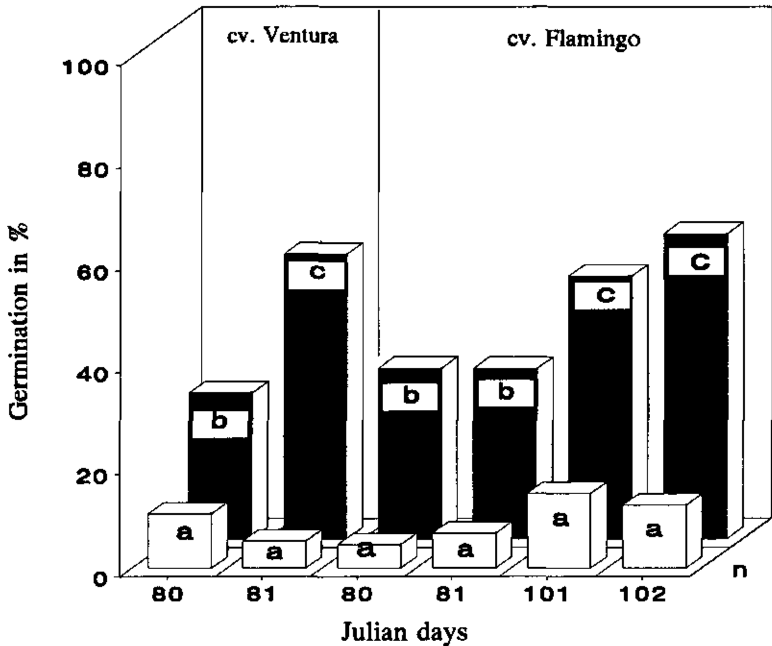



Figure 1. Spore germination of *V. lecanii* (in percent) observed on leaf disks from cv. Ventura and cv. Flamingo taken from glasshouse experiment 2 before (n) and after (i) 24 hours of incubation at high humidity. Entries are means of compartment and leaf level samples with 4 replications each. Different letters indicate significant differences ($P \leq 0.05$, ANOVA, LSD multiple range test).

In summary, before incubation at high humidity mean germination percentages of 7-12% of *V. lecanii* spores were found in the glasshouse according to counts on leaf disks stored in the refrigerator. After 24 hours incubation at near-saturation, mean germination percentages of about 33-56% were found, a highly significant ($P \leq 0.00001$) increase (Figure 1).

**Discussion and conclusions**

Since controls were free of *V. lecanii*, the isolation between glasshouse compartments and the procedures used to prevent cross contamination between compartments were effective with respect to *V. lecanii*. Incidental significant differences in cfu's between compartments point in the same direction, but require further explanation.

Differences were found between compartments. In experiment 1, more cfu's were isolated from compartment 5 than from 10, while in experiment 2 after treatments 2 and 3 more spores had germinated in compartment 7 than in 4. Since compartments 5 and 7 were situated at the northern side of the glasshouse and numbers 10 and 4 at the southern side, differences in insolation and energy household may have occurred which led to better survival of *V. lecanii* at the northern than at the southern side.

In experiment 2, significantly more spores had germinated after the third biocontrol treatment on cv. Ventura than on cv. Flamingo. After treatments 4 and 5 the percentage germinated spores on incubated leaf disks from cv. Flamingo reached the same level as on cv. Ventura after treatment 3. Possibly the amount of mildew, far more on cv. Ventura than on cv. Flamingo after treatment 3, has had a stimulating effect on the germination of *V. lecanii* spores.

*V. lecanii* failed to control powdery mildew in either of the two experiments. The density of *V. lecanii* spores seemed to be adequate but their germination in the glasshouse was low and in most places mycelium growth was negligible. The failure to germinate is attributed to lack of moisture. Two arguments support this view. First, high germination percentages and exuberant growth could be induced by incubation under near-saturated (>95% RH) conditions. Second, *V. lecanii* was growing luxuriantly on powdery mildew in the glasshouse where humid niches were available, as between two leaves touching each other or within the space formed by a curled leaf. In the experimental glasshouse used, designed at a semi-commercial scale, the atmosphere was too dry for *V. lecanii* to be effective. New formulations of *V. lecanii*, in combination with some humidity management in the glasshouse, might improve biocontrol of cucumber powdery mildew by *V. lecanii*.

# Chapter 8

## Effects of oil formulations on humidity requirements of *Verticillium lecanii* spores used in biocontrol of *Sphaerotheca fuliginea*

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Submitted

**Effects of oil formulations on humidity requirements of *Verticillium lecanii* spores used in biocontrol of *Sphaerotheca fuliginea***

**Abstract**

Biocontrol of cucumber powdery mildew, *Sphaerotheca fuliginea* (Schlecht.:Fr.) Poll., by *Verticillium lecanii* is seriously hampered at low humidities. The effect is especially marked at low humidity (60% RH) during the three hours following the application of *V. lecanii* spores suspended in water. Formulations of *V. lecanii* spores in oil might improve the situation. Arachid oil and two invert emulsions using either Sunspray 6N or paraffin oil were tested in formulations of *V. lecanii* spores. Arachid oil gave the best development of *V. lecanii* on mildewed cucumber leaves. *V. lecanii* formulated with arachid oil showed significantly better control of mildew than without. A concentration of 0.5% arachid oil was somewhat toxic to mildew but 0.05% was not. Arachid oil did not show toxicity to *V. lecanii*. The humidity requirements of *V. lecanii* formulated with and without 0.05% arachid oil were compared at 95, 90 and 85% RH. Arachid oil significantly reduced the humidity dependence of *V. lecanii*. Since arachid oil is safe for human consumption and not phytotoxic to cucumber leaves, low concentrations of arachid oil are recommended as an additive to increase the effectiveness of *V. lecanii* as a biocontrol agent of *S. fuliginea*.

**Introduction**

*Verticillium lecanii* has shown to be a potential biocontrol agent of cucumber powdery mildew (*Sphaerotheca fuliginea* (Schlecht.:Fr.) Poll.) (Askary *et al.*, 1997; Hijwegen, 1988; Verhaar *et al.* 1996; Verhaar *et al.*, 1997). High humidity conditions seemed to be of great importance to obtain good control. The humidity conditions at the plant surface are probably the most important factor influencing the germination, growth and survival of *V. lecanii* in this habitat (Verhaar *et al.*, accepted). Possibly, some of the variability of biocontrol results achieved with *V. lecanii* can be ascribed to this factor. When *V. lecanii* spores are sprayed onto the leaves in a water suspension, the evaporation of the water carrier may be so fast that insufficient time for germination in free water is available so that *V. lecanii* becomes

dependent on the humidity of the air. Because the humidity conditions in Dutch glasshouses are not optimal for biocontrol by mycoparasites, the humidity requirement of *V. lecanii* is still a bottleneck for commercial biocontrol.

Oil emulsions reduce dew dependence of mycoherbicides (Auld, 1993; Connick *et al.*, 1991). Formulations with oils could reduce the humidity requirements of *V. lecanii* too. Hora Oleo 11E and paraffin oil were already used as formulations in biocontrol experiments with mycoparasites on cucumber powdery mildew (Hijwegen, 1992; Philipp and Hellstern, 1986; Philipp *et al.*, 1990; Verhaar *et al.*, 1996) but did not give satisfactory results. For biocontrol of *S. fuliginea* formulations are needed that are not phytotoxic to cucumber leaves and provide moisture, possibly also nutrients, for the mycoparasites to germinate and infect the mildew.

Formulations of biocontrol agents can be liquid, powdery or granular. For biocontrol of powdery mildew liquid oil formulations are the most interesting. Because pure oils are toxic to temperate-zone plants by interfering with respiration and transpiration, oils must be applied at controlled dosage levels. This can be achieved by emulsification. Two methods are feasible. In one the water is the external phase and oil is the internal phase (oil in water). A range of vegetable oils was used as formulations of hyperparasites against insects (Bateman *et al.*, 1993). The use of low concentrations of vegetable oils with an emulsifying adjuvant was found to enhance efficacy of bioherbicides in the absence of dew in controlled environments (Auld, 1993; Klein, 1995).

In the other method the phases are reversed (invert emulsions). Recent research has shown the potential for invert emulsions for mycoherbicides by providing moisture for germination, enhancing the intensity of infections (Boyette *et al.*, 1991; Daigle *et al.*, 1990; Yang *et al.* 1993) and reducing the need to apply high dosages of inoculum (Amsellem *et al.*, 1990).

The aim of this study was to select a formulation which can improve the biocontrol potential of *V. lecanii*. In addition, we investigated the importance of high humidity during the first day after biocontrol treatment with *V. lecanii*.

## Materials and methods

### *Mildewed cucumber leaves*

Second and third leaves of three weeks old cucumber plants (*Cucumis sativus* cv Corona; De Ruiter Seeds, Bleiswijk, Holland), grown in a growth chamber (20°C; 16 hours light, 23 $W/m^2$ at leaf level, Pope FTL D32W/84HF; 70% RH), were

excised and immediately transferred to Petri dish units, each consisting of two superimposed Petri dishes, the lower one containing 50% Hoagland solution, the petioles reaching from the upper into the lower dish through a hole (Verhaar *et al.*, 1997). The Petri dish units were closed and placed in a growth chamber (20°C; 16 hours light, Pope FTL D32W/84HF; 70% RH) in low light intensity. After 24 hours the leaves were exposed to light and produced roots. About 10 days after the transfer of the leaves to the Petri dish units, the rooted leaves were inoculated with *S. fuliginea* in a vacuum-operated settling tower (Reifschneider and Boiteux, 1988) by interrupting a vacuum of -0.15 bar above four mildewed leaf disks (ø 1.5 cm), that were used as mildew inoculum. Per treatment, 24 rooted leaves could be inoculated simultaneously (Verhaar *et al.*, 1997). When necessary nutrient solution in the Petri dish units was replenished with 50% Hoagland solution.

Five days after the inoculation with mildew the lids of the Petri dish units were removed and leaves were sprayed with various fluids (treatments) by means of a De Vilbiss sprayer (Van der Kuip Ltd, Utrecht, The Netherlands), driven by compressed air, until drop formation (about 1.25 ml per leaf). After spraying, the mounted leaves were placed in climate cabinets (126x80x130cm, VEPHL 5/1350, Heraeus-Votsch Ltd., Frommern, Germany; 20 °C; 12 h light, TL 65/84 and 4x100W Ltd. Philips, Eindhoven, The Netherlands; temperature and humidity regulation by SRM-96 Jumbo Ltd., Weesp, The Netherlands; ultrasonic mist equipment EMS 2400 Ltd. Stulz, Amstelveen, The Netherlands; 2 $m^3$/hr fresh air flow; windspeed 0.2m/sec). A glass window-pane was placed 0.1m under the lamps to avoid damage to *V. lecanii* by ultraviolet light.

*Mycoparasite*

*Verticillium lecanii* (Zimm.) Viégas, strain Fyto 88.1, isolated from *S. fuliginea* (Hijwegen, 1988), was cultured for experiments I, II, IV and V on oatmeal agar (60gr oatmeal (HO, Quaker) and 20gr Agar Technical No.3 (Oxoid) in 1 l water), incubated at 20°C in the dark. For experiment III *V. lecanii* was cultured in a liquid medium of 3% milled, autoclaved oat meal (Verhaar and Hijwegen, 1993). Spores were harvested from two weeks old cultures by flooding the plates with 10 ml demineralized water or after one week of incubation from the liquid medium. Suspensions were filtered through cotton wool and adjusted to the desired density using a haemacytometer. To prepare the invert emulsions the spore suspensions were concentrated in a MSE Mistral refrigerated centrifuge at 3000 rpm.

*Experiments*

*Experiment I*. Rooted cucumber leaves were sprayed with a suspension of *V. lecanii* spores ($10^6$ spores/ml) and exposed to 7 different humidity regimes, indicated by the letters A to G. High humidity (H: near-saturated atmosphere) and low humidity (L: 60% RH) were alternated during 4 days as follows: H,L,H,L (A), H,H,L,L (B), L,L,H,H (C), L,H,L,H (D); L-H,L, H,L (E), L-H,H,L,L (F), L,L,L,L (G). In regimes E and F the leaves were exposed to low humidity during the first three hours. High humidity was obtained by placing the mounted leaves on grids in closed boxes (30 x 45 cm, 30-cm high), with 0.5 l of water at the bottom of the box. The low humidity was obtained by placing the mounted leaves in plastic boxes with some space between box and lid so that relative humidity in the box could adjust to that of the growth chamber (60% RH). Boxes were placed in a growth chamber (20°C, 16 h light, 23 W $m^{-2}$ at leaf level, Pope FTL D32W/84HF). After four days, two leaf disks (⌀ 1.5 cm) per leaf were taken and about 100 spores per disk were examined for germination.

*Experiment II*. Several vegetable oils as formulations of *V. lecanii* were tested for their effects on germination of *V. lecanii* spores and on biocontrol of *S. fuliginea* (Table 1). To test the effect on germination, a thin layer (1.5 ml) of formulated *V. lecanii* spores (5 x $10^6$ spores per ml) was incubated in a small Petri dish (⌀ 5 cm) at 20°C in the dark. After 24 hours the percentage of germinated spores was determined. Each treatment was conducted 4 times and the experiment was replicated once. The effect on biocontrol was tested on mounted mildewed leaves. Five days after mildew inoculation the mildewed leaves were sprayed with oil-formulated *V. lecanii* spores. Controls were sprayed with suspensions of *V. lecanii* without oil or with oil-containing formulations without spores. Treated leaves were placed on grids in closed plastic boxes (30 x 45, 30 cm high) in which 85% RH was obtained by means of a saturated solution of 0.5 l of NaCl at the bottom of the box (Verhaar *et al.*, accepted). Leaves treated with or without *V. lecanii* were placed in separate boxes to avoid cross-contamination. The boxes were placed in a growth chamber (20°C, 16h light, 23 $Wm^{-2}$ at leaf level (Pope FTL D32W/84 HF). After 9 days the effect on mildew was assessed.

*Experiment III*. Two invert emulsion carriers (IEC, water in oil type), containing an oil phase and a water phase (1:1, v/v), were prepared. In the first IEC the oil phase contained 98 ml of Sunspray 6N oil (kindly provided by Dr. S.-M. Yang, USDA-ARS, Frederick, USA) and 2 ml of Myverol 18-99 (a distilled monoglyceride emulsifier (Eastman Chemical Products, Inc. Kingsport, TN, USA, obtained via the courtesy of Barentz Grondstoffen B.V., Hoofddorp) after Yang *et al.* (1993). The

oil phase in the second IEC emulsion was prepared with 92 ml Paraffin oil (highly liquid, Merck Ltd, Darmstadt, Germany), 6 grams vaseline and 2ml Myverol 18-99. The water phase contained 0.02 ml Tween 80 (Merck-Schuchardt, Hohenbrunn bei München, Germany) per 100 ml tap water. The 50:50 water-in-oil emulsions were prepared by adding 10 ml of the water phase drop by drop to 10 ml of the oil phase in a 100 ml flask, which was continuously stirred for one hour by a magnetic stirrer. After that hour one ml of a spore suspension containing $10^{10}$ *V. lecanii* spores was added and the stirring continued for another 15 minutes. Because the 50:50 IEC emulsion based on Sunspray 6N was phytotoxic to cucumber leaves, the oil content in the IEC emulsions had to be reduced to 0.5%. Reduction from 50% oil contents to 5% was reached by gradually adding more of the water phase under continuous stirring. Finally the IEC emulsions were diluted to a concentration of 0.5% oil by pouring the 5% emulsion into water in a commercial laboratory Waring blender and blending for 2 minutes. The final spore concentration was $5x10^6$ spores per ml.

Arachid oil (Levo Ltd., Franeker, The Netherlands) formulations were prepared by stirring 10 ml or 1 ml arachid oil with 0.5 ml and 0.05 ml Tween 80, respectively, in 1 liter water on a magnetic stirrer. After two hours a spore suspension containing $10^7$ *V. lecanii* spores per ml was added (1:1/v:v), bringing the spore density to $5x10^6$ in the 0.5% arachid oil plus 0.025% Tween 80 (Exp. III-A) and the 0.05% arachid oil plus 0.0025% Tween 80 (Exp. III-B) formulations.

Experiment III was performed twice. Experiment III-A had 6 blocks with 3 leaves per treatment and experiment III-B had 4 blocks with 3 leaves per treatment. A randomized, complete block design was used. Assessments were made 10, 14, 17 and 19 (Exp III-A) and 9, 13, 16 and 19 (Exp III-B) days after the mildew inoculation. Leaves of one block were simultaneously inoculated with mildew and for each block spore suspensions and formulations of fresh *V. lecanii* were prepared. The relative humidity in the climate cabinets was adjusted to 90% RH.

*Experiment IV.* Experiment IV was performed as experiment III to compare the two arachid oil concentrations of 0.5% and 0.05%. This experiment was performed twice, following the design of Experiment III-B.

*Experiment V.* The effect of relative humidity on *V. lecanii* with or without arachid oil and with or without emulgator (0.001% Tween 80) was evaluated by adjusting the RH in the climate cabinets to 85%, 90% or 95% RH. The experiment was conducted in three blocks per humidity with 6 leaves per treatment. The humidity treatments were replicated three times. Assessments were made 7, 10, 12 and 14 days after mildew inoculations.

Table 1. Experiment II. Effect of oil formulations with or without emulgator on germination of *V. lecanii* and biocontrol effect of *V. lecanii* on *S. fuliginea*.

| Formulations of *V. lecanii* 5 x $10^6$ spores/ml | Stimulation of *V. lecanii* germination [d] | Improvement of biocontrol of *S. fuliginea* [e] | References |
|---|---|---|---|
| Water | - | - [c] | |
| Tween 80 [a] | - | - | |
| Lutensol A05 [a] | - | | |
| Paraffin [b] | - | + | 4 [f] |
| Maize oil | + | + | 3 |
| Sunflower oil | + | | 3 |
| Soy oil | + | | 3,4 |
| Olive oil | + | | 3 |
| Arachid oil | +++ | +++ | 1,3 |
| Paraffin [b] + Tween [a] | +++ | + | |
| Maize + Tween | ++++ | ++ | |
| Sunflower + Tween | + | | |
| Soy + Tween | ++ | | |
| Olive + Tween | + | | |
| Olive + Lutensol [a] | +++ | | |
| Arachid + Tween | +++ | ++++ | |
| Hora Oleo 11E | ++++ | +/- | 2,4,5 |

[a] Emulgator 0.05% (Tween 80, Merck-Schuchardt, Hohenbrunn bei München, Germany; Lutensol AO5, BASF, Ludwigshafen, Germany).
[b] Oil formulations 0.5%.
[c] Improvement compared to the water control recorded on a scale from - (no effect) to ++++ (excellent effect).
[d] Formulated *V. lecanii* spores were incubated for 24 hour at 20°C in the dark.
[e] Formulated *V. lecanii* sprayed on mildewed cucumber leaves incubated at 20°C and 85% relative humidity.
[f] 1) Auld (1993); 2) Hijwegen (1992); 3) Northover and Schneider (1993): 4) Philipp *et al.* (1990) and 5) Verhaar *et al.*, 1996.

*Assessments*

*Experiment I*. Leaf disks were colored by 0.1% fluorescent brightener 28 (Sigma) and examined at a magnification of 250x under a Zeiss Axioskop equipped with an incident-light fluorescence illumination, a 395-440 nm excitation filter, a 460 nm dichroic mirror and a 470 nm barrier filter. A spore was noted as germinated when the length of the germ tube exceeded the length of the spore. Per leaf disk the mean germ tube length was estimated and loosely expressed as a proportion of the spore length.

*Experiment II*. Spores were assessed for germination by the use of an inverted microscope. The percentage mildew attacked by *V. lecanii* was estimated. The effects of the formulations on germination and percentage of attacked mildew were compared to the water control and recorded on a scale from no effect (-), some effect (+), to excellent effect (++++).

*Experiments III, IV and V*. Per leaf the percentage mildewed leaf area was assessed using a stereo microscope with continuously variable magnification (8-50x) using the scale: 0, 0.1, 0.5, 1, 5, 10, 20, ..., 80, 90, 95, 99, 99.5, 99.9, 100. Additional visual observations were made of the mildew and of the mycoparasite under a light microscope (250x).

*Experiment V*. The amount of mildew inoculum was assessed by counting the number of mildew spores per $cm^2$ on a vaseline smeared object-glass placed on the bottom of the vacuum-operated settling tower before inoculation.

The humidity conditions on the leaf surface were calculated according to leaf energy balance and leaf transpiration equations after Goudriaan and van Laar (1994). Thereto, the temperatures on and above healthy cucumber leaves in the climate rooms with regulated RHs of 95, 90 and 85% were measured. Two thermocouples of 0.1 mm copperconstantane were pressed to the surfaces of two healthy cucumber leaves while the others were placed 5 cm above these leaves. For each humidity condition the temperatures were recorded by a datalogger during 24 hours.

*Data analysis*

The effect of RH regimes on germination percentages in Exp-I were analysed by ANOVA and mean percentages germination were compared by the Tukey multiple range test. Results of Experiment II were classified only, without statistical analysis. The control effects on total mildewed leaf area of Experiments III, IV and V were analyzed by ANOVA. The ANOVAs were applied to arcsine square root transformed data to improve homogeneity of error variances (Gomez and Gomez,

1984). The Tukey multiple range test was used for comparison of means on 19 days after mildew inoculation. Data presented in tables have been backtransformed. Longitudinal analyses (Zadoks, 1972) were made by comparison of the area under the mildew progress curves (AUMPCs) (Campbell and Madden, 1990). The AUMPCs were calculated from day 10 to day 19 (Exp III-A), day 9 to day 19 (Exp III-B and Exp IV) or day 7 to day 14 (Exp V), and analyzed by ANOVA.

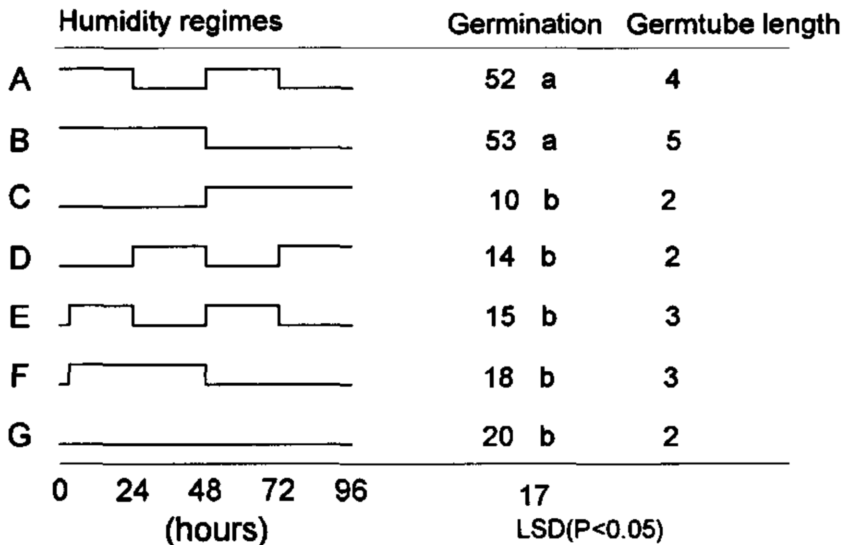



Figure 1. Experiment I. Germination of *V. lecanii* spores after different humidity regimes. Humidity regimes A to G are indicated by high and low lines (high = 99% RH; low = 60% RH). Germination percentages followed by different letters are significantly different (Tukey multiple range test, $P \leq 0.05$). Germtube lengths are estimated relative to spore lengths.

**Results**

*Experiment I.* The percentage germination of *V. lecanii* spores was significantly higher ($P \leq 0.05$) after regimes A and B than after the other regimes (Fig. 1). The average germ tube lengths were about 5 times the spore length after regime B, 4 times after regime A, 3 times the spore length after regimes E and F, and 2 times the spore length after regimes C, D and G.
*Experiment II.* Results indicated that vegetable oils (0.5%) can stimulate the development of *V. lecanii* at relative low humidity (85%) (Table 1). Arachid and maize oils with Tween stimulated the germination of *V. lecanii* and considerably improved the biocontrol potential of *V. lecanii* at reduced humidity. Oils from olive, soybean and sunflower, and paraffin and Hora Oleo 11E (a formulated paraffin oil) did not produce interesting results. None of the oils was toxic to cucumber leaves at the dosages used.
*Experiment III.* The water treated leaves showed the significantly highest ($P \leq 0.05$) AUMPCs in both experiments and highest mildew value in Exp III-A (Tables 2.1 and 2.2). The diluted IEC-oil formulations S (based on Sunspray 6N) and P (based on paraffin oil) showed a significant but modest reduction of mildew. A 0.5% arachid oil showed a significant and considerable control effect, but 0.05% arachid oil did not. *V. lecanii* alone gave variable control effects, modest in Exp III-B and considerable in Exp III-A. The differences between S and P on the one hand and *V. lecanii* on the other hand was marked in Exp III-A only. *V. lecanii* formulated with P gave significantly more control than P alone, but more control than *V. lecanii* alone only in Exp III-B. The effects of *V. lecanii* formulated with S and S alone did not differ significantly.

Microscopic observations after treatment with *V. lecanii* alone showed germinated spores all over the leaves but the hyphal networks of *V. lecanii* were mostly limited to places around leaf veins and hairs. After treatments with formulated *V. lecanii*, its hyphal networks were observed all over the mildewed leaves, especially if *V. lecanii* was formulated with arachid oil.
*Experiment IV.* Two concentrations of arachid oil (0.5 and 0.05%) to formulate *V. lecanii* spores were compared. Arachid oil in a concentration of 0.5% with *V. lecanii* gave better control than without, the difference being significant in Exp IV-B but not in Exp IV-A (Table 2.1 and 2.2). The 0.05% arachid formulation showed a different pattern. Arachid oil (0.05%) alone had no significant ($P>0.05$) control effect, whereas *V. lecanii* formulated with 0.05% oil showed a significantly

**Table 2.1 Experiments III and IV. Mildewed leaf area in percent, 9 days after biocontrol treatments.**

| Treatments | Experiments | | | |
|---|---|---|---|---|
| | Exp III-A | Exp III-B | Exp IV-A | Exp IV-B |
| Water | 96.7 f[y] | 94.4 e | 98.5 b | 96.7 d |
| S[x] | 56.3 de | 45.0 bc | | |
| P | 72.1 e | 64.3 cd | | |
| 0.5 A | 19.8 bc | | 19.2 a | 22.3 bc |
| 0.05 A | | 82.0 de | 91.3 b | 91.6 d |
| V | 11.4 b | 75.1 d | 78.0 b | 49.1 c |
| V+ S | 38.3 cd | 37.9 ab | | |
| V+ P | 31.6 bcd | 34.7 ab | | |
| V+ 0.5A | 0.5 a | | 3.4 a | 2.4 a |
| V+ 0.05A | | 20.8 a | 16.3 a | 7.3 ab |
| s.e.[z] | 4.8 | 4.2 | 7.1 | 4.8 |

[x] S = diluted IEC of 0.5% Sunspray, P = diluted ICE of 0.5% Paraffin oil, A = Arachid oil, V = *Verticillium lecanii* 5 x $10^6$ spores per ml. [y] Backtransformed arcsine transformed percentages of mildewed leaf area. In each column values followed by different letters are significantly different according to Tukey multiple range test ($P \leq 0.05$). [z] Pooled standard errors of the arcsine transformed values based on 6 (Exp III-A), 4 (Exp III-B) and 3 (Exp IV) replications.

**Table 3. Experiment V. Effect of biocontrol treatments and RH on the percentages mildewed leaf area, 9 days after the control treatments.**

| Treatments | RH's | | |
|---|---|---|---|
| | 85% | 90% | 95% |
| V + AT[x] | 61.9 a[y] | 22.9 a | 5.9 a |
| V | 75.1 ab | 38.6 b | 29.9 b |
| AT | 82.5 bc | 64.0 c | 44.1 c |
| W | 92.5 c | 71.0 c | 90.7 d |
| s.e. | 4.7[z] | 4.4 | 3.6 |

[x] V = *Verticillium lecanii* 5 x $10^6$ spores per ml, AT = Arachid oil formulation of 0.05% with 0.002% Tween 80, W = water. [y] Backtransformed arcsin transformed percentage of mildewed leaf area 9 days after the control treatments. In each column values followed by different letters are significantly different according the LSD multiple range test ($P \leq 0.05$). [z] Pooled standard errors of the arcsine transformed values based on 6 replications.

Table 2.2 Experiments III and IV. Areas Under the Mildew leaf area Progress Curves (AUMPC) from 5 to 14 days after biocontrol treatments.

| Treatments | Experiments | | | |
|---|---|---|---|---|
| | Exp III-A | Exp III-B | Exp IV-A | Exp IV-B |
| Water | 854 f[y] | 647 e | 772 b | 705 d |
| S[x] | 592 de | 235 abc | | |
| P | 451 e | 332 bcd | | |
| 0.5 A | 194 abc | | 102 a | 154 bc |
| 0.05 A | | 438 d | 604 b | 589 d |
| V | 101 ab | 385 cd | 466 b | 199 c |
| V+ S | 312 cd | 187 ab | | |
| V+ P | 247 bc | 196 ab | | |
| V+ 0.5A | 8 a | | 14 a | 15 a |
| V+ 0.05A | | 89 a | 69 a | 38 ab |
| s.e.[z] | 63 | 45 | 89 | 35 |

[x] S = diluted IEC of 0.5% Sunspray, P = diluted ICE of 0.5% Paraffin oil, A = Arachid oil, V = *Verticillium lecanii* 5 x $10^6$ spores per ml. [y] Area under the Mildewed Leaf Progress Curves from 5 to 14 days after treatments. In each column, values followed by different letters are significantly different according to Tukey multiple range test (P≤0.05). [z] Pooled standard errors; 6 (Exp III-A), 4 (Exp III-B) and 3 (Exp IV) replications.

(P≤0.05) better control than *V. lecanii* alone and kept the mildewed leaf area under 17%.

For the preparation of IEC-emulsions a very high initial spore concentration was required. Therefore we grew *V. lecanii* in liquid culture (Verhaar and Hijwegen, 1993) and concentrated the spores by centrifugation. We did not observe any difference due to the spore production method, either in the experiments reported here (compare *V. lecanii* plus arachid oil in Experiments III and IV) or in earlier unpublished experiments.

*Experiment V*. The mildew inoculum density varied from 130 to 690 spores per $cm^2$. No correlation was found between the density of mildew inoculum and the percentage of mildew or the mildew development expressed in AUMPCs. The overall ANOVA showed significant (P≤0.05) treatment and RH effects on the

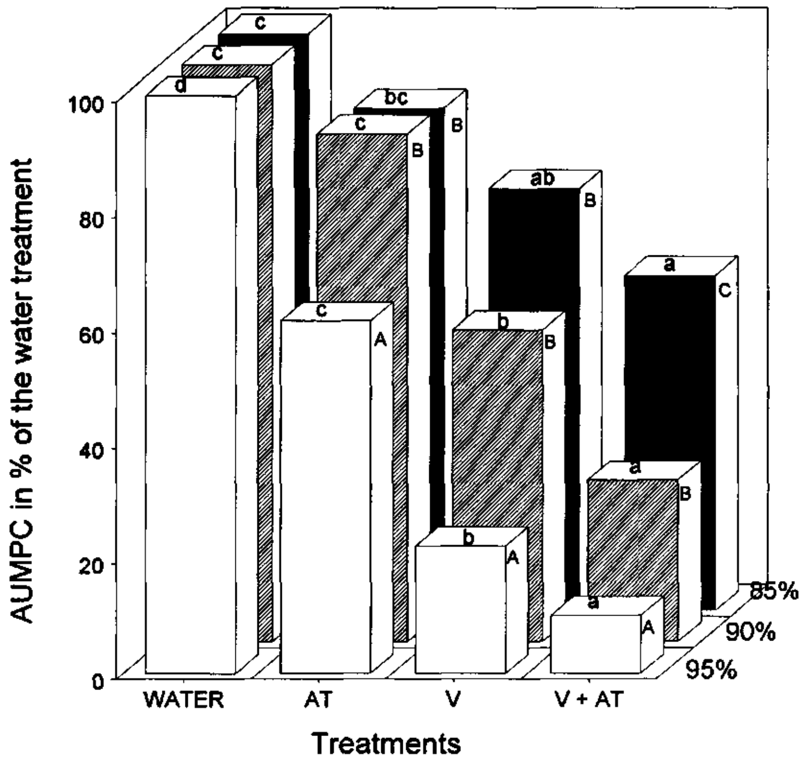



Figure 2. Experiment V. Effects of biocontrol treatments and RH on the Areas Under the Mildewed leaf Area Progress Curves (AUMPC). AUMPCs from day 2 to day 10 after biocontrol treatments, relative to the water controls (= 100%). The treatments were V = *Verticillium lecanii* 5 x $10^6$ spores per ml, AT = Arachid oil formulation of 0.05 % with 0.002% Tween 80 and the combination V + AT with 5 x $10^6$ spores per ml. In each row parallel to the treatment axis different lower case letters indicate significantly different AUMPCs between the biocontrol treatments. Different upper case letters in each row parallel to the RH axis indicate significantly different AUMPCs between the RH treatments. (LSD multiple range test $P \leq 0.05$). Pooled standard errors were AT, 7; V, 10; V + AT, 6, and 85%, 7; 90%, 7; 95, 9, based on 6 replicates.

percentages of mildewed leaf area 9 days after treatment and a significant interaction between these factors. The treatment effects and the differences between the control treatments were most pronounced at 95% RH (Table 3). At humidities of 85% and 90% the results of the arachid treatment (AT) did not differ significantly from those of the water treatment (W). *V. lecanii* with and without arachid oil gave significant ($P \leq 0.05$) control effects at all humidities. Addition of arachid oil to *V. lecanii* (V + AT) produced considerably better control than *V. lecanii* alone (V), the differences being significant ($P \leq 0.05$) at 90 and 95% but not at 85% RH. Humidity had a significant effect ($P \leq 0.05$) on all treatments (Fig. 2).

The mean differences in temperature between the leaves and air temperature at 95%, 90% and 85% RH were 1.2, 1.4 and 1.6°C, respectively. According to the leaf energy balance and leaf transpiration calculations the humidities on the leaf surface were 93%, 89% and 85% (Appendix). These figures have indicative value only.

## Discussion

The rooted leaf system (Verhaar *et al.*, 1997, accepted) is a useful method for laboratory tests of biocontrol treatments. AUMPCs from day 5 to 14 after biocontrol treatment on cucumber showed the same pattern as the mildewed leaf areas at day 9. So, for rough screenings of biocontrol treatments only one assessment of mildewed leaf areas at day 9 will give enough information.

Humidity regimes of alternating high and relative low RH have an enormous influence on the development of *V. lecanii.* A RH of 60% for three hours following the application of *V. lecanii* spores resulted in a substantial reduction of percentage germinated spores and of germtube growth (Experiment I). High humidity seems to be especially important during the first hours after biocontrol treatment with unformulated *V. lecanii.* Appropriate timing of the application in the late afternoon and the use of formulations can possibly address the problem posed by lack of humidity.

In preliminary experiments two invert emulsions with 50% oil phase gave good mildew control but they were highly phytotoxic to cucumber leaves (Verhaar, unpublished). In the present study we diluted the IEC-emulsions to 0.5% oil hoping to create a water-oil-water emulsion. Though invert-emulsions were reported to have excellent qualities for the formulation of mycoherbicides (Yang *et al.*, 1993), their application in the biocontrol of fungi such as powdery mildews is questionable.

First, phytotoxicity can be accepted for mycoherbicides but not for a fungicide on a valuable crop such as cucumber. Second, complete coverage of the target organism is necessary for good control of powdery mildew on cucumber, but not for good control of a weed. Thus a large amount of formulation is required, resulting in high costs and environmental pollution. Because an invert emulsion containing 5% oil phase is still a sticky semi-solid, special equipment may be needed for experimentation with invert emulsions.

For biocontrol of powdery mildew invert-emulsions offer little promise. Formulations with arachid oil give interesting control results, and arachid oil is biodegradable and non-polluting.

The 0.5% arachid oil without *V. lecanii* gave significant control, whereas 0.05% did not (Experiments III and IV). Several oils have antifungal properties. Calpouzos (1966) reviewed efficacy of oils for the control of fungal diseases such as powdery mildews. The antifungal properties against powdery mildews were recently demonstrated by several researchers (Häberle and Schlösser, 1993, Horst *et al.*, 1992; Northover and Schneider, 1993; Northover and Schneider, 1996; Ohtsuka and Nakazawa, 1991; Pasini *et al.*, 1997; Schneider and Northover, 1991). Oils can cause deformation of conidia, inhibition of germination (Ohtsuka and Nakazawa, 1991) and inactivation of mildew lesions by attack of mycelium. Northover and Schneider (1996) suggested that vegetable oils may have significant action against vine powdery mildew only in pre-lesion treatments and as antisporulantia in treatments applied to established lesions.

In biocontrol there is no demand for formulation chemicals which are fungitoxic by themselves. Low concentrations of vegetable oils as formulation agents may improve control results. Arachid oil (0.05%) favours the germination, growth and sporulation of *V. lecanii*. Supposedly, a slight damage to *S. fuliginea* makes nutrients available to *V. lecanii* and thus gives *V. lecanii* a better opportunity to attack the mildew.

Several investigators (Frampton and Longree, 1941; Jhooty and McKeen, 1964; Weinhold, 1961) favour the hypothesis that the humidity at the leaf surface, which is supposed to be higher than in the ambient air, accounts for the development of fungi on leaves. As the measurement of humidity at the leaf surface is technically difficult we estimated it by calculation. The calculated humidity at the leaf surface during experiment V was about the same as or even lower than the humidity in the air. Because these RHs are not favourable for *V. lecanii* development, other factors such as the powdery mildew itself, leaf exudates or the

phyllosphere microflora could have stimulated *V. lecanii* development in Experiment V.

In conclusion, a 0.05% arachid oil has potential as formulation of *V. lecanii* for biocontrol on cucumber powdery mildew. It is safe for human consumption and biodegradable, shows no phytotoxicity to cucumber leaves, has little fungitoxicity to *S. fuliginea*, reduces RH dependence of *V. lecanii* and increases the effectiveness of *V. lecanii* to control cucumber powdery mildew.

## Appendix

According to the leaf energy balance and leaf transpiration equations elaborated by Goudriaan and van Laar (1994, chapter 7) the following equation is derived:

$$R_{v\ leaf\ surface} = \frac{R_{v\ air} + (\gamma s r_b R_n/Pc_p)/((s+\gamma^*)e_s) + \gamma(1-R_{v\ air})/(s+\gamma^*)}{1 + (S r_b R_n \gamma^*/Pc_p)/((s+\gamma^*)e_s) - s/(s+\gamma^*)(1-R_{v\ air})}$$

where

$Pc_p$ Volumetric heat capacity of air, 1200 J/m$^3$ °C

$\gamma$ is the Psychrometer constant = 0.67 mbar/°C

s slope of the saturated vapor pressure curve (hPa°C$^{-1}$) at 20°C about 1.5 mbar/°C

$r_b$ Leaf boundary layer resistance to heat s/m, at open stomata about 100 s/m

$\gamma^*$ $\gamma^* = \gamma(r_b + r_s)/r_b$

$r_s$ Leaf stomatal resistance s/m, at open stomata about 100 s/m

$R_n$ Net radiation absorbed per leaf area W/m$^{-2}$

$R_n = \lambda E + H$

where

H is sensible heat loss per leaf area

$H = \Delta t\ Pc_p/r_b$

$\lambda E = (sH + \delta)/\gamma^*$

$\delta = D_a\ Pc_p/r_b$

$D_a$ = Vapor pressure deficit of air

$e_s = 6.107 \exp(17.4\ T_{air}/(239 + T_{air}))$

# Chapter 9

# Sensitivity of the mycoparasite *Verticillium lecanii* to fungicides used against *Sphaerotheca fuliginea* on cucumber

M.A. Verhaar

T. Hijwegen

# Sensitivity of the mycoparasite *Verticillium lecanii* to fungicides used against *Sphaerotheca fuliginea* on cucumber


## Abstract

The effect of fungicides, registered in the Netherlands for control of *Sphaerotheca fuliginea* on cucumber, on the mycoparasite *Verticillium lecanii* was tested. Tolerance of *V. lecanii* for triforine and pyrazophos allows an integration of biological and chemical control.


In glasshouse experiments the potential of the mycoparasite *Verticillium lecanii* (Zimm.) Viégas strain Fyto 88.1 for the control of cucumber powdery mildew (*Sphaerotheca fuliginea* (Schlecht.:Fr.) Poll.) was tested (Verhaar *et al.*, 1996; Dik *et al.*, 1998). *V. lecanii* did not satisfactorily control mildew on the susceptible cucumber cultivars Corona and Ventura. Therefore the biocontrol treatments had to be replaced by fungicide treatments (fenarimol, bupirimate or imazalil) when disease became severe. After treatments with bupirimate we observed *V. lecanii* growing abundantly on the affected mildew (Verhaar, unpublished). Supposedly, *V. lecanii* parasitizes mildew suffering from fungicide damage more easily than healthy mildew. If so, integrated control, here a combination of biological and chemical control, may decrease the amount of fungicides applied in cucumber production. To investigate the tolerance of *V. lecanii* for fungicides registered in the Netherlands for the control of cucumber powdery mildew, their effect on mycelial growth of *V. lecanii* was tested on agar plates.

Growth of *V. lecanii* was determined *in vitro* on malt agar with three different concentrations of fungicides (1, 10 and 100 ppm active ingredients) and on agar without fungicides (controls). The fungicides used were bitertanol (pure, Bayer BV, Mijdrecht, the Netherlands), bupirimate (Nimrod® (25% bupirimate), Zeneca Agro, Ridderkerk, the Netherlands), fenarimol (pure, Imex-Hulst BV, Hulst, the Netherlands), imazalil (pure, Janssen Pharmaceutica NV, Beerse, Belgium), pyrazophos (pure, Hoechst Holland NV, Amsterdam, the Netherlands), tolylfluanide (Euparen® (50% tolylfluanide), Bayer BV, Mijdrecht, the Netherlands) and triforine (pure, Imex-Hulst BV, Hulst, the Netherlands). The pure fungicides were suspended in 1 ml ethanol (70%) and the formulated products in sterilised water before mixing with 40 ml of molten malt agar (Oxoid). Per fungicide concentration two dishes were filled with 20 ml medium. The mycoparasite *V. lecanii* was cultured on oatmeal agar (60 g oatmeal and 20 g Agar Technical (Oxoid) in 1 l water) in Petri dishes at 20 °C

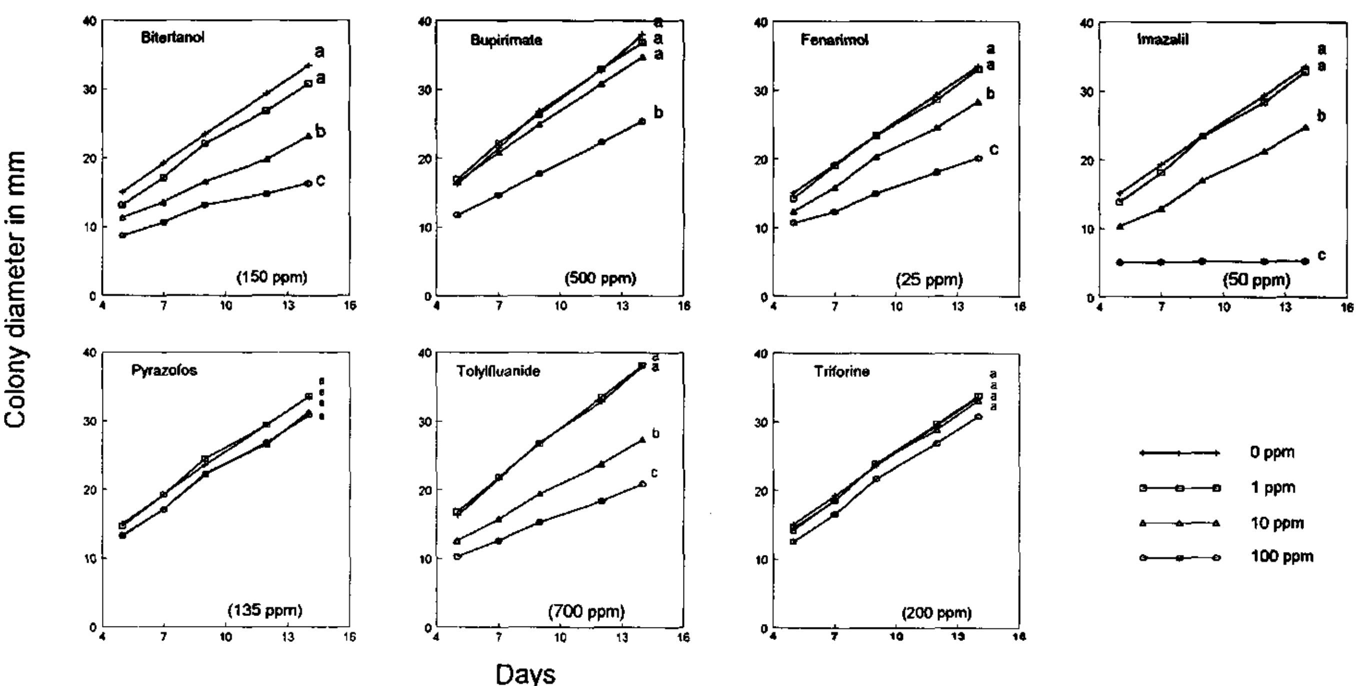



Figure 1. Mean diameter in mm of the *V. lecanii* colonies growing on malt agar with fungicides (+ = no fungicide; □ = 1 ppm; △ = 10 ppm, ○ = 100 ppm) versus time in days. Lines followed by different letters have significantly ($P \leq 0.05$) different regression coefficients (= mean radial colony growth rates) according to the LSD-test. Per fungicide the concentration used in practice against *S. fuliginea* is placed within brackets.

in darkness. Three plugs of 2mm diameter of two week old *V. lecanii* cultures were placed upside down on the agar surface in an triangle with sides of 4.5 cm. The inoculated plates were incubated at 20°C in darkness. Radial growth was measured 5, 7, 9, 12 and 14 days after inoculation by measuring the diameters of the cultures. The experiment was performed in two replicated blocks. A Petri dish was handled as the experimental unit. The mean radial growth rate of the three colonies per Petri dish was calculated by linear regression of colony diameter on time (mm/d). Radial growth rates per fungicide were analyzed by ANOVA and compared by LSD-test.

Growth of *V. lecanii* on the seven fungicides is presented in figure 1. Per fungicide there were no significant ($P \leq 0.05$) block - concentration interactions. *V. lecanii* showed significantly ($P \leq 0.05$) different growth rates on the various concentrations of bitematol, fenarimol, imazalil and tolylfluanide. No growth of *V. lecanii* was recorded on 100 ppm imazalil. On 100 ppm bupirimate the growth rate of *V. lecanii* was significantly reduced. Pyrazophos and triforine had no effect on the growth rates of *V. lecanii*, conform Saito and Yabuta (1996). In their experiments, pyrazophos and triforine caused only 8 and 2% reduction, respectively, of mycelial growth of *V. lecanii* at a concentration of 100 ppm. With 1000 ppm they found reductions of 28% and 72%, respectively. The concentrations of active ingredients used in practice are well below 1000 ppm, about 135 ppm and 200 ppm, for pyrazophos and triforine, respectively. Contrary to our findings, Hall (1981b) observed a high percentage inhibition by pyrazophos of *V. lecanii* conidiospore germination and mycelial growth. Such differences point to differences in sensitivity to fungicides between strains of *V. lecanii*.

Studies on compatibility of fungicides and biocontrol agents in tritrophic systems were made by Ravensberg *et al.* (1994) with *V. lecanii* on whiteflies and aphids and by Sundheim (1982) with *Amplomyces quisqualis* on cucumber powdery mildew. Their results also pointed to differential effects among fungicides on interference with biocontrol. For example, triforine, safe in our experiments, interfered with *V. lecanii* for insect control but not with *A. quisqualis* for mildew control.

We observed our most promising biocontrol strain Fyto 88.1 of *V. lecanii* to be compatible with 10 ppm triforine applied to mildewed leaves (compare Sundheim, 1982). Together they showed a better control effect than each separately (Table 1, Chapter 10). The tolerance of *V. lecanii* to triforine and pyrazophos *in vitro* should be explored to arrive at an integrated control of powdery mildew on greenhouse cucumber by a combination of biological and chemical means.

# Chapter 10

# General discussion

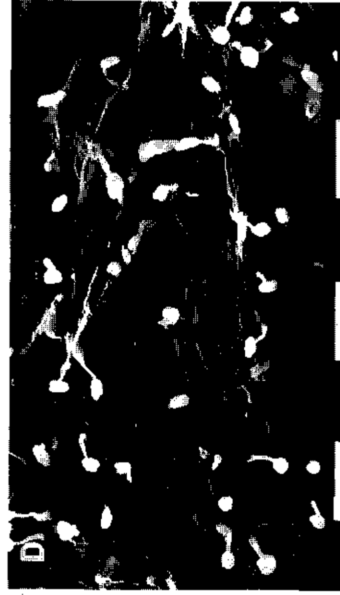
## General discussion

The aim of the research presented in this thesis was to investigate the potential of biocontrol by mycoparasites on cucumber and rose powdery mildews. Thereto bioassays for two tritrophic systems had to be developed. Methods were developed based on rooted cucumber leaves and detached young rose leaves. The tritrophic cucumber system seemed much easier to study than the tritrophic rose system, because of differences in behaviour of the two systems. As rose leaves become rapidly resistant to powdery mildew, only young leaves with just unfolded leaflets are susceptible (Wheeler, 1978; Frinking and Verwey, 1989). Furthermore, in glasshouses a maximum of about 5 colonies per rose leaflet was observed, and the colonies reached a maximum size of 5 to 10 mm (Chapter 2). In contrast, mildew colonies on rooted cucumber cv. Corona leaves kept growing until the whole leaf was covered by powdery mildew. Treated with 2,6-dichloroisonicotinic acid the susceptible rose cv. Madelon behaved like the partially resistant cucumber cv. Flamingo (Hijwegen and Verhaar, 1994; Hijwegen *et al.*, 1996). These differences in development between the rose and cucumber powdery mildews on susceptible rose and cucumber cultivars, respectively, make the tritrophic cucumber system preferable. The rooted leaf technique (Chapters 5, 6 and 8) for the tritrophic system cucumber, powdery mildew and mycoparasite offered a good method to study this system for about three weeks.

In the first exploratory glasshouse experiments (Chapter 4) *V. lecanii* controlled cucumber powdery mildew better than *S. rugulosa*, with or without Hora Oleo 11E formulation. Askary *et al.* (1998) suggested that Hora Oleo 11E had worked against *S. rugulosa*. However, we observed stimulation of *S. rugulosa* on agar plates with the addition of Hora Oleo 11E in comparison to agar plates without (unpublished data). In combination with the use of a partially resistant cucumber cultivar the biocontrol of *S. fuliginea* by *V. lecanii* showed to have prospects. We assume that mycoparasites can more easily destroy slowly growing mildew colonies on partially resistant plants than fast growing mildew on susceptible cultivars.

Following preliminary experiments on biocontrol of cucumber powdery mildew by different mycoparasites (unpublished data), the results of *V. lecanii* on rose powdery mildew (Chapter 2), and the promising results of *V. lecanii* in our first glasshouse experiments (Chapter 4) and considering that this fungus was already used against whitefly in the Netherlands, most research was focused on *V. lecanii*. Under favourable circumstances (> 95% r.h. and 20°C), *V. lecanii* showed considerable reduction in mildewed leaf area after early preventative (9 and 5 days

B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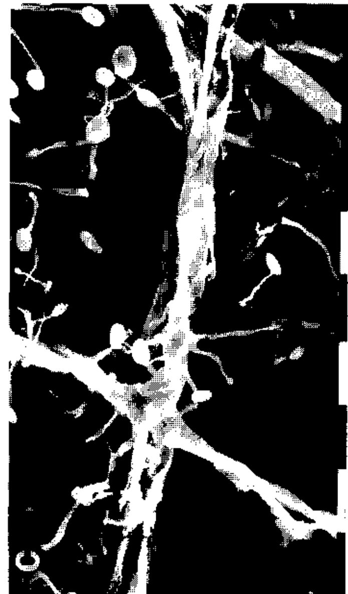
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before mildew inoculation) and early control treatments (2 days after mildew inoculation) (Chapter 6). This result suggested that an early start of biocontrol treatments might be important to achieve good control.

As high humidity was reported to be necessary for the infectivity of *V. lecanii* against insects (Hsiao *et al.*, 1992) and for the mycoparasitic capacity on *S. pannosa* (Chapter 2), the behaviour of a group of *V. lecanii* isolates at different humidities was studied in an attempt to select *V. lecanii* isolates with a high biocontrol capacity (Chapter 5). Non of the tested developmental stages, germination, growth rate and sporulation, provided a good clue for selection of isolates with high biocontrol potential. The *V. lecanii* isolate (Fyto 88.1), taken in 1988 from *Sphaerotheca fuliginea* in the glasshouse of the Laboratory of Phytopathology at Wageningen showed good mycoparasitic behaviour but intermediate scores for the developmental stages tested (Chapter 5). Putative host specificity and other characters such as mycelium growth type and production of enzymes to attack the powdery mildew hyphae might have more predictive value. A correlation was found between parasitism of powdery mildew and lysis of *Cladosporium cladosporioides* growing in dual culture on agar with *V. lecanii* isolates. So, *C. cladosporioides* might offer a suitable alternative substrate to test isolates of *V. lecanii* isolates for mycoparasitic potential (Chapter 5). The results with a *V. lecanii* isolate of Mycotal®, chapter 5, indicated that *V. lecanii* isolates are host specific, but Askary *et al.* (1998) described a *V. lecanii* isolate which was virulent to aphids and antagonistic to cucumber powdery mildew. Selection of *V. lecanii* isolates with wide host ranges might be interesting for biocontrol in glasshouses.

Figure 1. External observations of interactions between *V. lecanii* and *S. fuliginea* by Scanning Electron Microscopy (SEM). For cryofixation pieces of cucumber leaves with parasitized cucumber powdery mildew were mounted on brass stubs, subsequently frozen in liquid nitrogen, transferred to a cryochamber (Hexland CT 1000/CP 2000, Oxford Instruments, Oxford, UK), sublimated at 90 °C and 0.1 Pa for 30 min and sputter coated with gold for 2 min. Samples were examined with a scanning electron microscope (Philips SEM 535, Eindhoven, The Netherlands) at 15kV accelerating voltage.
Bars are 10µm. After 3 days *V. lecanii* hyphae grew in close contact with powdery mildew hyphae which collapsed and shrivelled (A). *V. lecanii* formed appressoria like structures (B, arrows). Six days after biocontrol treatment most powdery mildew hyphae were destroyed (C & D).

At optimal conditions we observed *V. lecanii* to develop abundant mycelium growing around and in close contact with mycelium, hyphae and conidia of *S. pannosa* or *S. fuliginea* (Chapters 2, 5, 6 and 8). Scanning electron microscopy (SEM) was used for external observation of the interaction between *S. fuliginea* and *V. lecanii*. *V. lecanii* grows in close contact with *S. fuliginea* and after a while the powdery mildew mycelium collapsed and shrivelled (Figure 1A). Appressoria-like structures of *V. lecanii* were observed (Figure 1B). Six days after control treatment *V. lecanii* grew on and through the moribund hyphal cells (Figure 1C), which resulted in a total destruction of the powdery mildew (Figure 1D). Askary *et al.* (1997) observed attachment of *V. lecanii* to *S. fuliginea*, mechanical pressure, degrading cell walls, penetration and active growth of *V. lecanii* inside *S. fuliginea* by transmission electron microscopy. By cytochemical labelling of chitin Askary *et al.* (1997) observed production of chitinases and they hypothesized that a complex strategy involving production of cell-wall degrading enzymes and diffusion of antifungal metabolites, causing disintegration of the cytoplasm, precedes parasitism and internal colonization.

In glasshouse experiments at a semi-commercial scale in the Research Station of Glasshouse Vegetables at Naaldwijk *V. lecanii* showed unexpectedly poor control results, in contrast to our preliminary glasshouse experiments (Chapter 4). While considerable amounts of vital *V. lecanii* spores were present on the cucumber leaves during the whole experiment just a small percentage germinated in the glasshouse (Chapter 7 and Appendix). Differences in environmental conditions, plant conditions and used formulations could have caused the differences between glasshouse experiments at the two locations. Temperatures were comparable at both locations. The highest level of r.h. in Wageningen was higher (99%) than in Naaldwijk (80% or 90%) (Chapters 4 and 7). In Wageningen the cucumber plants were growing in the soil and the plants were watered once a day by wetting the soil. This treatment might have caused a higher r.h. in Wageningen than in Naaldwijk where plants were growing on rockwool. In addition, the nutrient supplies were probably different between the two cultivation systems (soil and rockwool). Van Andel (1952) described the influence of salt concentrations in the nutrition on the rates of exudates formation for tomato plants. The formulations used in Wageningen were Hora Oleo 11E and pure water, while Tween and white oil were used in first and second glasshouse experiments in Naaldwijk, respectively. During the glasshouse experiments in Naaldwijk differences in amounts of *V. lecanii* cfu's isolated from leaf disks and in percentages of germinated *V. lecanii* spores were

found between glasshouse compartments at the north and south side (Appendix Chapter 7). Insolation and energy household might have influenced the biocontrol agent *V. lecanii*.

The biocontrol activity of *Sporothrix flocculosa*, which showed the best control results against *S. fuliginea* of the three tested biocontrol agents (*Ampelomyces quisqualis, S. flocculosa* and *V. lecanii*), appeared to be based on antibiotics (Hajlaoui *et al.*, 1992; Hajlaoui and Bélanger, 1993). Death of the pathogen occurred rapidly without cell wall erosion or penetration. In view of these observations we question the designation of *S. flocculosa* as mycoparasite.

During the glasshouse experiments our method to produce phialoconidia of *V. lecanii* on liquid oat meal suspension (Chapter 3) showed to be very efficient, while no differences were observed between spores of *V. lecanii* that originated from Petri dishes or from liquid medium (Chapter 5).

It must be concluded that current conditions in 'commercial' glasshouses are not optimal for biocontrol by *V. lecanii*. Especially the r.h. during the first hours after biocontrol treatment seemed to be very important (Chapter 8). Manipulation of the fungus or the environmental conditions possibly offer *V. lecanii* more chances. Possibly physiological approaches, such as modifications of waterstress and C:N ratios in growth media (Magan, 1998) may support *V. lecanii*. Environmental conditions can be improved by misting water over the crop. High humidity in cucumber and rose glasshouses, however, may stimulate other fungal pathogens such as *Botrytis cinerea*.

Table 1. Mean percentage mildewed leaf area on rooted cucumber leaves, after integrated control by 0.01N triforine and *V. lecanii*, either treatment alone and a water control (n = 6) 5, 7 and 10 days after control treatments at 20°C and 90% r.h.

| Days after treatments | water control | *Verticillium lecanii* | Triforine (0.01N) | Triforine + *V. lecanii* |
|---|---|---|---|---|
| 5 | 28 b[a] | 3 a | 7 a | 1 a[b] |
| 7 | 67 b | 7 a | 8 a | 4 a |
| 10 | 78 b | 22 a | 25 a | 11 a |

[a] Values in rows followed by the same letter do not differ significantly at $P \leq 0.05$ in ANOVA.
[b] Excluding the water control from ANOVA the combined treatment differed significantly ($P \leq 0.05$) from the single treatment at day 5.

The microclimatic conditions on the leaves can be influenced by formulations. Arachid oil reduced the r.h. dependence of *V. lecanii* and increased the effectiveness of *V. lecanii* on cucumber powdery mildew (Chapter 8).

During the glasshouse experiments in Naaldwijk we observed *V. lecanii* growing abundantly on fungicide treated mildew. Possibly a combination of fungicide and biocontrol agent or alternating applications of these two offer another alternative to the use of fungicide alone. Thereto the effect of fungicides, registered in the Netherlands for the control of *S. fuliginea*, on the growth of the mycoparasite *V. lecanii* was tested. The tolerance of *V. lecanii*, isolate Fyto 88.1, for triforine and pyrazophos allows an integration of biological and chemical control. Preliminary experiments were done to find suitable concentrations of fungicide to study the potential of integrated control of the fungicide triforine and the biocontrol agent *V. lecanii*. An indication of the cumulative effect of 0.01N triforine and *V. lecanii* ($10^6$ spores/ml) is given in Table 1. *V. lecanii* should colonize mildew weakened by fungicide more easily than healthy mildew.

Results generated in this thesis have shown that *V. lecanii* has potential as a biocontrol agent of powdery mildew on roses and cucumber. However, the process of mycoparasitism takes a few days and biocontrol by *V. lecanii* is seriously hampered at low humidity. Possible improvements such as using partially resistant cucumber cultivars, preventive biocontrol, formulations of *V. lecanii* spores in oil and integration of biocontrol and fungicidal control need further exploration.

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# Summary


Chapter 1. Rose and cucumber are valuable Dutch export products. Intensification of these crops has facilitated the occurrence of powdery mildew on rose (*Sphaerotheca pannosa*) and cucumber (*Sphaerotheca fuliginea*). Intensive control by fungicides is needed, since resistant cucumber cultivars are sensitive to chlorosis and poorly adapted to use in Dutch glasshouses and resistant roses are rarely used. A new tendency is to grow greenhouse vegetables and flowers in an environmentally friendly way. Good results have been obtained with biocontrol of insects. In almost all cucumber glasshouses one or more insect species are biologically controlled. The demand for non-chemical control of fungal diseases is growing. Thereto a great diversity of alternative control treatments, such as water, plant extracts, oils, antitranspirants, salts, composts and biological agents were tested on powdery mildews of cucumber and rose. Nonetheless, intensive control by fungicides is still needed. In the scope of the Multi-Year Crop Protection Plan, published by the Dutch government and aiming at a drastic reduction of the use of fungicides, an additional research program was set up. The project investigated biocontrol of powdery mildew on cucumber and rose by means of mycoparasites.

Chapter 2. A bioassay was developed to study the effectiveness of mycoparasites to control powdery mildew on roses under selected environmental conditions. One isolate of *Ampelomyces quisqualis*, *Sporothrix rugulosa* and *Tilletiopsis minor*, and four isolates of *Verticillium lecanii* were tested at four relative humidities (r.h.), 100, 90, 80 and 70% at 20°C. Each of these fungi seemed to have a humidity dependent pattern of mycoparasitism. Most mycoparasites lost their effectiveness rapidly below 100% r.h., but two *V. lecanii* isolates showed mycoparasitic activity at 90% r.h. One isolate achieved over 80% mildew control at 90% r.h., 6 days after biocontrol treatment.

Chapter 3. As efficient inoculum production of *Verticillium lecanii* was needed for biocontrol experiments at the glasshouse scale, a method was developed for efficient production of phialoconidia of *V. lecanii*. When *V. lecanii* was cultured in a 3% milled oat meal suspension at 135 rpm in the dark at 25°C for 7 days, over $3 \times 10^9$ phialoconidia/ml were produced.

Chapter 4. Two potential biological control agents of cucumber powdery mildew (*Sphaerotheca fuliginea*), *Verticillium lecanii* and *Sporothrix rugulosa* were tested under glasshouse conditions. Two experiments were carried out. In both experiments the control treatments began by spraying whole plants one week after the first observation of mildew on inoculated leaves. In the first experiment, two cucumber cultivars with different levels of resistance, cv. Corona (susceptible) and cv. Flamingo (partially resistant), were used. *V. lecanii* controlled the mildew better than *S. rugulosa*. On cv. Flamingo, *V. lecanii* could keep the mildew severity below 15% infected leaf area for 9 weeks after inoculation with *S. fuliginea*. Treatment by Hora Oleo 11E, a formulated paraffin oil, alone or as an additive to *V. lecanii* were as good as the fungicide treatments. In the second experiment, weekly treatments with *V. lecanii* on cv. Flamingo kept the mildew severity at a level below 20% infected leaf area during 10 weeks. Weekly and biweekly treatments with Hora Oleo 11E again gave good control. These glasshouse experiments demonstrated that biocontrol of *S. fuliginea* in combination with the use of a partially resistant cucumber cultivar has some prospect. Selection for *V. lecanii* isolates with a high biocontrol potential and a search for appropriate formulations might improve the efficiency of *V. lecanii*.

Chapter 5. To identify characteristics for the selection of *Verticillium lecanii* with high potential for biocontrol of *Sphaerotheca fuliginea* under glasshouse conditions, the effect of water limitation on the development of 14 isolates of *V. lecanii* was explored. Conidial germination, growth and sporulation of isolates of *V. lecanii* were studied in a tritrophic system on cucumber leaves and *in vitro* in Petri dishes. The mycoparasitic ability of these isolates was studied in a tritrophic system on *S. fuliginea* and *in vitro* on *Cladosporium cladosporioides*. All characters were clearly affected by humidity. Four isolates showed good biocontrol potential. The performance of isolates *in vitro* had less predictive value than in the tritrophic system. Germination of isolates of *V. lecanii* was lower and mycelial growth faster on agar than on mildewed leaves under corresponding humidity conditions. This result suggests that conditions in the phyllosphere differed from the set humidity in the ambient air. A correlation was found between lysis of *C. cladosporioides* growing on agar with parasitism of powdery mildew on detached, rooted leaves. *C. cladosporioides* might offer a suitable substrate for testing isolates of *V. lecanii* for mycoparasitic potential. Conidial germination, growth and sporulation had limited predictive value for mycoparasitic potential. Two isolates performed well according to most components measured, while isolate 1 (used

in our previous work) had an intermediate position although its overall performance was rather good. Putative host specificity and other characters, such as branching pattern of the mycelium, might have more predictive value. Experiments with tritrophic systems showed to be complex and vulnerable to disturbances which could not always be identified. Growth of *S. fuliginea* and *V. lecanii* largely occur in the boundary layer of the cucumber leaves, about 50µm in depth. Little is known about the temperature and humidity in the boundary layer relative to their set values.

Chapter 6. The effect of timing the application of the mycoparasite, *Verticillium lecanii*, on cucumber powdery mildew was studied in a rooted cucumber leaf bioassay. The mycoparasite was applied at different times (day -9, -5, -2, -0, +2, +5, +9 relative to the day of mildew inoculation (day 0)). At high humidity (> 95% r.h.), early preventative (day -9 and -5) and early curative control treatments (day +2) gave considerable reduction in mildewed leaf area, while late curative treatments resulted in more mildewed leaf area but ultimately in a reduced amount of healthy mildewed leaf area (<20%). Spores of *V. lecanii* deposited near leaf veins and trichome bases, probably places with high humidity and good nutrient supply, began to grow rapidly, and within one week the fungus developed mycelial networks with good local sporulation. The good control results of the early treatments suggest that *V. lecanii*, growing saprophytically, needs at least 5 days to develop to the stage where it can successfully parasitize young powdery mildew hyphae. Good timing of biocontrol treatment by *V. lecanii* seems to be important to achieve good control.

Chapter 7. In cooperation with the Research Station for Floriculture and Glasshouse Vegetables in the Netherlands the effect of three biocontrol agents, *Ampelomyces quisqualis*, *Sporothrix flocculosa* and *Verticillium lecanii* was tested on cucumber powdery mildew (*Sphaerotheca fuliginea*). Two glasshouse experiments, one in the summer and one in winter/spring were conducted on a semi-commercial scale. In both experiments, a susceptible and a partially resistant cultivar were used. In the second experiment, the additional effect of integration of biological control and silicon amendments to the nutrient solution was assessed. In both experiments, *A. quisqualis* did not control the disease. *V. lecanii* had a small effect on powdery mildew in the first experiment but not in the second. *S. flocculosa* gave the best control of powdery mildew in both experiments. In the first experiment, weekly application of *S.*

*flocculosa* reduced disease in the partially resistant cultivar to the same level as a treatment in which the fungicides bupirimate and imazalil were each applied once. Addition of silicon to the nutrient solution in a concentration of 0.75nM reduced disease by 10-16%, averaged over all treatments. There was no interaction between silicon and the biocontrol agents. Yield, recorded in the second experiment, was significantly increased by the fungicide and the *S. flocculosa* treatments compared to the control in the partially resistant cultivar. Silicon has no effect on yield in either cultivar.

Appendix chapter 7. Some detailed observations on the fate of *Verticillium lecanii* were made which might explain the failure of *V. lecanii*. The development of *V. lecanii* in the glasshouse was studied. The influence of glasshouse compartment, cucumber cultivar and leaf level was determined on the number of cfu's and the percentage germinated *V. lecanii* spores on leaf disks sampled 12 hours or one week after biocontrol. In both experiments more cfu's were isolated from the compartment at the northern side than from the southern side. Possibly, differences in insolation and energy household led to better survival of *V. lecanii* at the northern side. In experiment 2, significantly more spores had germinated after the third biocontrol treatment on cv. Ventura (susceptible cultivar) than on cv. Flamingo (partial resistent cultivar). After biocontrol treatments 4 and 5 in experiment 2 the percentage germinated spores on incubated leaf disks from cv. Flamingo reached the same level (around 50%) as on cv. Ventura after treatment 3. Possibly, the amount of mildew had a stimulating effect on the germination. The density of *V. lecanii* spores on the cucumber plants in the glasshouse seemed to be adequate but their germination was low and in most places the mycelial growth was negligible. As high germination and exuberant growth of *V. lecanii* could be induced by incubation at high humidity and *V. lecanii* was observed to grow on the mildew only at some humid niches in the glasshouse, the failure of *V. lecanii* to control powdery mildew was attributed to low humidity.

Chapter 8. The study intended to find a formulation which can improve the biocontrol potential of *Verticillium lecanii*. In addition, the importance of high humidity during the first day after biocontrol was investigated. The effect of alternating high and low relative humidity on development of *V. lecanii* was tested on rooted cucumber leaves sprayed with *V. lecanii* spores. High humidity seems to be important for the development of *V. lecanii* during the first hours after application. A r.h. of 60% for

three hours following the application of *V. lecanii* resulted in a substantial reduction of spore germination and germtube growth. Several vegetable oils to formulate *V. lecanii* were tested for their effects on germination of *V. lecanii* spores and on biocontrol of *S. fuliginea*. Arachid and maize oils (0.5%) with Tween (0.05%) stimulated the germination of *V. lecanii* and improved its biocontrol potential. Arachid oil and two invert emulsions using either Sunspray 6N or paraffine oil were tested in formulations of *V. lecanii* spores. Because of phytotoxicity, high costs and environmental pollution, invert emulsions offer little promise for biocontrol of fungi. Arachide oil (0.5%) gave better results than invert emulsions diluted to 0.5%. *V. lecanii* formulated with arachid oil showed significantly better control of mildew than without. A concentration of 0.5% arachid oil was somewhat toxic to cucmber powdery mildew but 0.05% was not. Arachid oil did not show toxicity to *V. lecanii*. The humidity requirements of *V. lecanii* formulated with and without 0.05% arachid oil were compared at 95, 90 and 85% r.h. Arachid oil significantly reduced the humidity dependence of *V. lecanii*. Since arachid oil is safe for human consumption and not phytotoxic to cucumber leaves, low concentrations of arachid oil are recommended as an additive to increase the effectiveness of *V. lecanii* as a biocontrol agent of *S. fuliginea*.

Chapter 9. Observations of *Verticillium lecanii* growing on cucumber powdery mildew in glasshouses, where fungicides were applied against cucumber powdery mildew, led to the idea of integrated control of biocontrol and fungicides. Thereto the tolerance of *V. lecanii* to fungicides registered in the Netherlands for the control of cucumber powdery mildew was investigated. Pyrazophos and triforine had no effect on the growth rates of *V. lecanii*. *V. lecanii* was compatible with 10 ppm triforine applied to mildewed leaves. Together they showed a better control effect than separately (Chapter 10). The tolerance of *V. lecanii* to triforine and pyrazophos *in vitro* should be explored to arrive at an integrated control of powdery mildew on greenhouse cucumber by a combination of biological and chemical means.

Chapter 10. Results generated in this thesis showed that *Verticillium lecanii* has potential as a biocontrol agent of powdery mildew on roses and cucumber. Mycoparasitism, however, takes time and biocontrol by *V. lecanii* is seriously hampered at low humidity. Possible improvements such as the use of partially resistant

cucumber cultivars, preventative biocontrol and formulations of *V. lecanii* spores in vegetable oil were discussed.

## Samenvatting

Hoofdstuk 1. Roos en komkommer zijn succesvolle Nederlandse export producten. Door het intensiveren van deze teelten wordt echte meeldauw op roos (*Sphaerotheca pannosa*) en komkommer (*Sphaerotheca fuliginea*) een steeds groter probleem. Omdat resistente komkommer-gewassen gevoelig zijn voor chlorose en resistente rozen niet vaak gebruikt worden, is in de teelt van rozen en komkommers een intensieve bestrijding van echte meeldauw met fungiciden noodzakelijk. Het telen van groenten en bloemen in kassen op een milieuvriendelijke manier wordt de laatste jaren steeds populairder. Goede resultaten worden behaald met biologische bestrijding van insekten. Nu wordt in bijna alle komkommerteelten één of meer soorten insekten biologisch bestreden. De vraag om niet-chemische bestrijdingsmethoden voor schimmelziekten neemt toe. Hoewel een verscheidenheid aan alternatieve bestrijdingsmiddelen zoals water, plant-extracten, olies, antitranspiranten, zouten, compost en biologische bestrijding werden getest tegen echte meeldauw op komkommer en roos is intensieve chemische bestrijding nog steeds nodig. In het kader van het Meerjarenplan Gewasbescherming, waarin de Nederlandse regering een drastische reductie van het gebruik van fungiciden nastreeft, werd het in dit proefschrift beschreven project uitgevoerd. Een onderzoek werd verricht naar de bestrijding van roze- en komkommer-meeldauw met behulp van mycoparasieten.

Hoofdstuk 2. Een toets werd ontwikkeld om het effect van verschillende mycoparasieten op echte meeldauw van roos te testen onder verschillende klimatologische omstandigheden. Eén isolaat van *Ampelomyces quisqualis*, *Sporothrix rugulosa* and *Tilletiopsis minor* en vier isolaten van *Verticillium lecanii* werden getoetst bij vier relatieve luchtvochtigheden (r.v.), 100, 90, 80 en 70%, bij 20°C. De schimmels vertoonden r.v.-afhankelijke patronen van mycoparasitisme. De meeste mycoparasieten verloren hun effectiviteit snel beneden een r.v. van 100%, maar twee *V. lecanii* isolaten vertoonden ook mycoparasitisme bij een r.v. van 90%. Eén van deze isolaten kwam tot een bestrijding van meer dan 80% bij een r.v. van 90%.

Hoofdstuk 3. Omdat voor kasproeven een efficiënte productiemethode van *Verticillium lecanii* nodig was werd een methode ontwikkeld voor efficiënte productie van phialoconidiën van *V. lecanii*. Een productie van meer dan 3 x $10^9$ phialoconidiën werd bereikt nadat *V. lecanii* 7 dagen gekweekt werd in een roterende (135 rpm) vloeibare oplossing van 3% gemalen havermout bij 25°C in het donker.

Hoofdstuk 4. Twee candidaten voor biologische bestrijding van echte komkommer meeldauw ( *Sphaerotheca fuliginea*), *Verticillium lecanii* en *Sporothrix rugulosa*, werden getoetst onder kascondities. Twee experimenten werden uitgevoerd. In beide experimenten werd met de biologische bestrijding gestart een week nadat de eerste ziekteverschijnselen zichtbaar waren op de geïnoculeerde bladeren. In het eerste experiment werden een vatbaar komkommerras (cv. Corona) en een partieël resistent ras (cv. Flamingo) gebruikt. *V. lecanii* bestreed de meeldauw beter dan *S. rugulosa*. Op cv. Flamingo hield *V. lecanii* de meeldauw aantasting gedurende 9 weken na meeldauwinoculatie onder de 15% aangetast bladoppervlak. Behandeling met Hora Oleo 11E (geformuleerde paraffine-olie) alleen of als toevoeging aan *V. lecanii* gaf een vergelijkbare bestrijding als de fungicide-behandeling. In het tweede experiment werden wekelijkse en tweewekelijkse behandelingen met *V. lecanii* op cv. Flamingo uitgevoerd. Bij een wekelijkse behandeling met *V. lecanii* bleef het aangetaste bladoppervlak 10 weken lang beneden de 20%. Wekelijkse en twee-wekelijkse behandelingen met Hora Oleo 11E gaven opnieuw goede bestrijding. Deze kasexperimenten lieten zien dat de combinatie van biologische bestrijding en het gebruik van een partiëel resistente komkommer-cultivar mogelijkheden biedt. Selectie van *V. lecanii* isolaten met een groot vermogen om echte meeldauw te bestrijden en het gebruik van een juiste formulering zal mogelijk de efficiëntie van *V. lecanii* kunnen verbeteren.

Hoofdstuk 5. Om eigenschappen te selecteren van belang voor goede biologische bestrijding van *Sphaerotheca fuliginea* in de kas werd het effect van vochtgebrek op de ontwikkeling van 14 *V. lecanii* isolaten bestudeerd. Kieming van conidiën en groei en sporulatie van *V. lecanii* werden bestudeerd in een tritroof systeem en *in vitro* in Petri-schalen. Mycoparasitisme werd getoetst in een tritroof systeem op meeldauw en *in vitro* op *Cladosporium cladosporioides*. Alle getoetste eigenschappen werden door vochtgebrek beïnvloed. Vier isolaten van *V. lecanii* vertoonden goede biologische bestrijding. Het gedrag van de isolaten *in vitro* had minder voorspellende waarde dan het gedrag van de isolaten in het tritrofe systeem. *In vitro* waren de kiemingspercentages lager en de groeisnelheden hoger dan onder vergelijkbare vochtcondities op bemeeldauwde komkommerblaadjes. Dit resultaat suggereert dat de condities in de fyllosfeer verschilden van de ingestelde waarde van de r.v. Een correlatie werd gevonden tussen de lysis van *C. cladosporioides* groeiend op agar en de parasitering van echte meeldauw groeiend op bewortelde komkommerblaadjes. *C.*

*cladosporioides* biedt mogelijk een goed alternatief om *V. lecanii* isolaten te toetsten op hun parasiterend vermogen op schimmels. Kieming van conidiën, groei en sporulatie hadden een beperkte voorspellende waarde voor het vermogen tot mycoparasitisme. Twee isolaten van *V. lecanii* scoorden goed bij de diverse eigenschappen, terwijl isolaat 1 (gebruikt in onze voorgaande experimenten) een gemiddelde positie innam maar over het algemeen genomen niet slecht naar voren kwam. Gastheer-specificiteit en eigenschappen zoals het vertakkingspatroon van het mycelium zullen misschien meer voorspellende waarde hebben. Experimenten met tritrofe systemen zijn complex en gevoelig voor verstoringen die niet altijd makkelijk geïdentificeerd kunnen worden. De groei van *S. fuliginea* en *V. lecanii* speelt zich in de fyllosfeer af in een laagje van 50µm. Weinig is bekend over de temperatuur en relatieve luchtvochtigheid op het blad ten opzichte van de ingestelde waarde in de omringende lucht.

Hoofdstuk 6. Het effect van het tijdstip van biologische bestijding met *Verticillium lecanii* werd bestudeerd op met meeldauw geïnfecteerde bewortelde komkommer blaadjes. De mycoparasiet werd toegediend op verschillende tijdstippen (dag -9, -5, -2, -0, +2, +5, +9 ten opzichte van de dag van meeldauw-inoculatie (dag 0)). Bij hoge luchtvochtigheid (≥95%) veroorzaakte vroeg preventieve (dag -9 en -5) en vroeg curatieve (dag +2) behandelingen een aanzienlijke reductie van meeldauw, terwijl na latere curatieve behandelingen de blaadjes meer meeldauw aantasting lieten zien, waarvan uiteindelijk het grootste gedeelte door *V. lecanii* aangestast bleek te zijn (>80%). *V. lecanii* sporen die bij bladnerven en bladharen terecht waren gekomen groeiden relatief snel uit en ontwikkelden binnen een week mycelium met goede sporulatie. De goede resultaten behaald met vroege behandeling suggereren dat *V. lecanii*, wanneer deze saprofytisch groeit, ten minste 5 dagen nodig heeft voordat het met succes jonge hyfen van echte meeldauw kan aantasten. Goede timing van biologische bestrijding met *V. lecanii* lijkt belangrijk voor het verkrijgen van een goed bestrijdingsresultaat.

Hoofdstuk 7. In samenwerking met het Proefstation voor de Bloemisterij en Glastuinbouw in Naaldwijk werd het effect van drie biologische bestrijdingsorganismen, *Ampelomyces quisqualis*, *Sporothrix rugulosa* en *Verticillium lecanii* op komkommermeeldauw (*Sphaerotheca fuliginea*) getoetst. Twee kasproeven, één in de zomer en één in winter/voorjaar werden uitgevoerd op semi-commerciële

schaal. In beide proeven werden een vatbaar en een partiëel resistent komkommerras gebruikt. In de tweede proef werd het effect van een combinatie van biologische bestrijding met silicium-toevoegingen aan het voedingsmedium getoetst. In beide proeven had *A. quisqualis* geen effect. *V. lecanii* vertoonde een klein effect in het eerste experiment. *S. flocculosa* gaf in beide experimenten het beste resultaat. In de eerste proef werd met een wekelijkse *S. flocculosa* behandeling op de partiëel resistente komkommer-cultivar een bestrijdingseffect verkregen vergelijkbaar met het effect van twee fungicide behandelingen. De toevoeging van 0.75nM silicium veroorzaakte een gemiddelde ziekte-reductie van 10-16%. Er was geen interactie tussen silicium en mycoparasiet. In de tweede proef was de opbrengst bij de met fungicide en *S. flocculosa* behandelde planten hoger dan die van de onbehandelde partieël resistente cultivar. Silicium had geen effect op de opbrengst.

Appendix bij hoofdstuk 7. Enkele gedetailleerde waarnemingen werden gedaan om het lot van *Verticillium lecanii* in de kassen te bestuderen en zo mogelijk het falen van de biologisch bestrijding met *V. lecanii* te verklaren. De invloed van kascompartiment, komkommer-cultivar en bladlaag op de aanwezigheid van vitale *V. lecanii*, het aantal cfu's en het percentage gekiemde sporen een week en 12 uur na de laatste biologische bestrijding werd getoetst. In beide proeven werden meer cfu's geïsoleerd van compartimenten aan de noordzijde dan aan de zuidzijde. Verschillen in instraling en energiehuishouding hebben mogelijk geleid tot een betere overleving van *V. lecanii* aan de noordzijde. In proef 2 werden na de derde biologische bestrijdingsbehandeling meer gekiemde sporen op de vatbare cultivar Ventura dan op de partiëel resistente cultivar Flamingo gevonden. Na de vierde en vijfde behandeling bereikte het percentage gekiemde sporen op cv. Flamingo het zelfde niveau (ongeveer 50%) als op cv. Ventura na de derde behandeling. Mogelijk heeft de hoeveelheid echte meeldauw een stimulerend effect gehad op de kieming van *V. lecanii*. In de kassen bleek de dichtheid van *V. lecanii* sporen op de komkommerplanten voldoende maar de kieming en myceliumgroei was op de meeste plaatsen te verwaarlozen. Aangezien hoge kieming en uitbundige groei van *V. lecanii* geïnduceerd konden worden door incubatie bij hoge luchtvochtigheid en in de kas op enkele vochtige plaatsen op de meeldauw goed groeiende *V. lecanii* werd aangetroffen kan het falen van *V. lecanii* toegeschreven worden aan een te lage relatieve luchtvochtigheid.

Hoofdstuk 8. Deze studie had tot doel om een formulering te zoeken die het vermogen van *V. lecanii* als biologisch bestrijdingsmiddel zou kunnen verhogen. Daarnaast werd

het belang van hoge relatieve luchtvochtigheid (r.v.) bestudeerd gedurende de eerste dag na toediening van *V. lecanii*. Het effect van wisselend hoge en lage luchtvochtigheid werd getoetst op bewortelde komkommerblaadjes die met een sporensuspensie van *V. lecanii* werden bespoten. De eerste uren na toediening leek de luchtvochtigheid grote invloed te hebben op de ontwikkeling van *V. lecanii*. Een luchtvochtigheid van 60% gedurende drie uren na toediening veroorzaakte een aanzienlijke teruggang in sporenkieming en kiembuisgroei. Verscheidende plantaardige olies werden als formulering van *V. lecanii* getoetst op hun invloed op kieming en biologische bestrijding van *Sphaerotheca fuliginea*. Aardnoot- en maïsolie (0.5%) met Tween (0.05%) stimuleerden de kieming en verhoogden het bestrijdend vermogen van *V. lecanii*. Aardnoot-olie en twee invert-emulsies gebaseerd op Sunspray 6N of paraffine-olie werden getoetst in formuleringen met *V. lecanii* sporen. Door fytotoxiciteit, hoge kosten en vervuiling van de omgeving lijken invert-emulsies niet goed bruikbaar in de biologische bestrijding van schimmels. Aardnoot-olie (0.5%) gaf betere resultaten dan tot 0.5% verdunde invert-emulsies. *V. lecanii* geformuleerd met aardnoot-olie gaf significant betere meeldauw bestrijding dan zonder formulering. Een concentratie van 0.5% aardnoot-olie was een beetje toxisch voor echte meeldauw maar 0.05% was dit niet. Aardnoot-olie was niet toxisch voor *V. lecanii*. De vochtafhankelijkheid van geformuleerde en niet geformuleerde *V. lecanii* werd vergeleken bij 95, 90 en 85% r.v. Aardnoot-olie reduceerde de vochtafhankelijkheid significant. Omdat aardnoot-olie veilig is voor mensen en niet fytotoxisch is voor komkommerbladeren worden lage concentraties aardnoot-olie aanbevolen als toevoeging aan *V. lecanii* om de effectiviteit van dit biologische-bestrijdingsorganisme tegen *S. fuliginea* te verhogen.

Hoofdstuk 9. Waarnemingen van *Verticillium lecanii* groeiend op komkommermeeldauw na fungicide-behandelingen in kasproeven leidden tot het idee van integratie van biologische en chemische bestrijding. Daarvoor werd de tolerantie van *V. lecanii* getest voor fungiciden die in Nederland tegen komkommermeeldauw gebruikt worden. Pyrazofos en triforine hadden geen remmend effect op de groeisnelheid van *V. lecanii in vitro*. *V. lecanii* bleek compatibel met 10 ppm triforine op bemeeldauwde blaadjes. Beide middelen samen vertoonden een betere bestrijding dan ieder apart (Hoofdstuk 10). De tolerantie van *V. lecanii* voor triforine en pyrazofos *in vitro* zou verder onderzocht moeten worden om te komen tot een geïntegreerde bestrijding van echte meeldauw op komkommer met een combinatie van chemische en biologische middelen.

Hoofdstuk 10. De resultaten van het onderzoek besproken in dit proefschrift laten zien dat *Verticillium lecanii* mogelijkheden heeft als biologisch bestrijdingsmiddel van echte meeldauwen op komkommer en roos. Mycoparasitisme van echte meeldauw door *V. lecanii* kost echter tijd en de biologische bestrijding wordt sterk gehinderd bij lage luchtvochtigheid. Verbetering van het resultaat door gebruik van partiëel resistente komkommer-rassen, preventieve biologische bestrijding en formuleringen met plantaardige oliën werd besproken.

**Curriculum Vitae**

Maria Anna (Marjan) Verhaar werd op 9 februari 1960 geboren te Amsterdam. In 1979 behaalde ze haar VWO diploma op het College Hageveld te Heemstede. Daarna volgde ze twee jaar een dansopleiding aan de Theaterschool te Amsterdam. In september 1981 begon zij een studie Biologie aan de Universiteit van Amsterdam. Met het hoofdvak fytopathologie en de bijvakken informatica en didactiek studeerde ze in 1988 af. Tijdens het laatste jaar van haar studie presenteerde ze natuurwetenschappelijke onderwerpen in het televisie programma 'Het Klokhuis'. Het schooljaar 1988/1989 was ze werkzaam in het voortgezet onderwijs als docente biologie. Bij de vakgroep Theoretische Biologie van de Vrije Universiteit van Amsterdam werkte ze van september 1989 tot juni 1991 als assistent docent mee aan de ontwikkeling van een cursus bioinformatica. In juni 1991 aanvaarde ze een baan als Assistent In Opleiding aan de Landbouwuniversiteit Wageningen. April tot juli 1997 volgde ze bij Ceva IT te Wageningen een opleiding tot Oracle specialist. Nu is ze werkzaam als programmeur/systeemanalist bij het Nederlands Rundvee Syndicaat te Arnhem.

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* The indicated publications are included in this thesis.