EAST MALLING RESEARCH

Report to: GlaxoSmithKline/HDC research fund
c/o James Wickham
Nine Oaks
Harpers farm
Goudhurst
Kent TN17 1JU

Tel: 01580 211127

EMR Contract Manager Mr Ian Hardie
East Malling Research
East Malling
West Malling
Kent ME19 6BJ

Tel: 01732 843833
Fax: 01732 849067

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IPM methods for blackcurrant gall mite and leaf midge, 2005.

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J V Cross
East Malling Research

Principal Scientists

J. V. Cross PhD, MA, MBPR, FRES (Study Director)
Réné Van Wezel (Author of report)
A Harris MSc

Authentication

I declare that this work was done under my supervision according to the procedures described herein and that this report is a true and accurate record of the results obtained.

Signed……..
J. V. Cross (Study director)

Dated……

East Malling Research is an officially recognised efficacy testing organisation (Certification No. ORETO 043)

Summary

A laboratory experiment (Phytotron unit, 20ºC, 95%RH) was conducted in spring 2005 to establish whether blackcurrant gall mite is susceptible to parasitisation by *Lecanicillium longisporum (*formerly named *Verticillium lecanii*), in particular whether infection of mites inside galls or emerging from galls can be caused by dipping galls in a aqueous suspension of spores. Treatments tested were Vertalec (0.2%) (A), Vertalec plus the adjuvant Codacide oil (0.3%) (B), adjuvant only (C) and untreated (D). Mites emerging from galls were captured in miniature sticky traps and examined for mycosis. At the end of the experiment after 2 weeks, the galls were cut open and the mites inside examined and tested for infection. The mite trapping data showed that 9 days after the start of the experiment, the treatments with the adjuvant Codacide oil (B and C) had reduced numbers emerging compared to the Vertalec alone (A) or the untreated control (D). No fungus resembling *L. longisporum* was found on the emerged mites at the outset, but putative positive samples were found 3 days after the start of the experiments in treatments A, B and C and in the untreated material after 9 days. Inside the galls, the Vertalec (A) and untreated control (D) were mostly fungus free, but the treatments containing Codacide oil (B and C) showed considerable amounts of fungus parasitizing the mites. Examination of these mites showed mycelia nearly always formed around mites, even when the body of the mite had been degraded. Fungal penetration via the mouth parts did occur but in very low numbers, and was not been found to be *L. longisporum.* Putative *L. longisporum* mycelium was found within the buds in treatments B and C only, and then only in low quantities. The results suggested that the Vertalec strain of *L. longisporum* is not pathogenic enough to be used as a control agent against blackcurrant gall mite.

Introduction

A three-year (2003-2006) DEFRA-funded research project (HH115TSF) aims to develop effective IPM methods for blackcurrant gall mite and leaf midge. Objective 1.4 of the project is to determine whether foliar sprays of the commercially available strain of the entomopathogenic fungus *Verticillium lecanii* (now named *Lecanicillium longisporum*), Vertalec (Koppert UK), can be exploited as a biocontrol agent of gall

mite and identify the optimum time of year when it should be applied. Field trials in the first years of the project tested multiple spray programmes of Vertalec throughout the growing season. However, fungal infection of mites within galls due to the multiple spray treatments could not be demonstrated. It was not clear whether this was due to failure of the pathogen to penetrate the galls, a lack of pathogenicity of the fungus to the mite or because weather conditions were not conducive to infection during the field trails. Here we report the results of a laboratory experiment in spring 2005 to investigate bicontrol of the mites in controlled conditions in the laboratory.

Materials and methods

Plant material

Sixty blackcurrant stem cuttings of the Ben Lomond variety were collected from East Malling Research plots on 14 April 2005. At this time migration of the gall mite up the stems had begun but had not yet reached 50% of the total migration.

The stems were trimmed to leave some leaves and 1 swollen bud (gall) per cutting. Each stem was cut 3 cm above the bud, and double-sided sticky tape was applied around the stem for the trapping of mites, leaving the gall to migrate up the stem.

The plant cuttings were kept in bottles with tap water at constant temperature, light conditions (20°C, light:dark 14:10hr) and a relative humidity of 95% in a calibrated Phytotron unit.

Treatments

Four days after start of the experiment, the sticky tape was removed and the number of mites counted. The cuttings were divided over 4 treatments: Vertalec treated (A), Vertalec plus adjuvant (Codacide oil) (B), adjuvant only (C) and untreated (D).

A Vertalec suspension was made with tap water and left for 3 hrs at ambient temperature according to the manufacturers' instructions (Koppert, UK). The treatments were applied on 19 April 2005 by dipping the stems in a 0.2% suspension of Vertalec (treatment A), or with the addition of 0.3% Codacide oil (treatment B), or

0.3% Codacide oil alone (treatment C). The untreated stems were dipped in tap water only (D).

The sticky tapes (with trapped mites) were changed every 3-4 days, and fixed to microscope slides (mites facing up). After counting the mites, sticky tapes from 6 of the cuttings (out of 15 for each treatment) were mounted directly for microscopical examination (I), another 6 were incubated on wet filter paper in Petri dishes for 3-4 days (until next sticky tape change) (II), and the remaining 3 tapes were similarly incubated for 6-8 days before mounting (III).

Six days after first application (25 April), treatments were repeated for part of the material: cuttings 1-3, 7-9 and 13-15. All cuttings were incubated for a further week.

Microscopy and Assessments

Two weeks after first treatment the experiment was ended, the blackcurrant gall was removed aseptically and the mites counted. The outer leaves were removed and the remainder of the gall was quartered and incubated separately on (semi) selective media (water agar; SDAY; SDAY+dodine). The last quarter section of the bud was used to directly observe mites. Mites were sampled by pressing sticky tape against part of the bud. These sticky tapes were mounted directly in lactophenol with aniline blue stain and left for at least two weeks before fungal assessment.

A sample of approximately 10 mites from each bud (if present) was mounted on a microscope slide using double-sided sticky tape, so that internal infection of the mites by fungi could be investigated.

Identification of *L. longisporum* and examination of infestation of mites by the fungus was achieved using a compound microscope with medium to higher power optics (x400). Double-sided (transparent) sticky tape for sampling gave adequate results, both for examination and counting of trapped mites from blackcurrant stems and for sampling individual mites and the associated fungus from the inside of buds at the end of the experiment.

The pieces of double-sided tape were mounted on standard microscope slides (sample side facing up), flooded with mounting medium and sealed with a glass coverslip, ensuring a thinly mounted slide. The slightly opaque background resulting from the sticky tape is visible in the images but this does not impair the examination of the fungus.

Freshly made lactophenol stain (20g phenol crystals, 20ml lactic acid, 40ml glycerol made up to 100 ml with water) with either 0.05% methylene blue or aniline (cotton) blue was used as a mounting medium in order to clear the mites and stain the fungus. Slides were left for at least two weeks before examination, in order to give ample time for clearing of the mites. Methylene blue only slightly stained fungal hyphae and did not stain *L. longisporum* spores at all. For this reason most of the slides were mounted in lactophenol with aniline blue. Stained fungi were compared with *L. longisporum*, grown directly from a Vertalec spore suspension (positive control) on Sabouraud dextrose agar plates (SDAY, 10 g/l peptone, 40 g/l glucose, 2 g/l yeast extract, 15 g/l agar).

Results

Mite migration data

Mites that migrated from the gall were intercepted at the tip of the stem by the sticky tape. The migrating mites increased in number in the adjuvant treatments (B Vertalec+Codacide oil, C Codacide oil only) 7 days after the treatments were applied. Mite migration data from treatment A (Vertalec only) approximately followed that of the untreated stems (D), albeit on a lower level (Fig. 1).

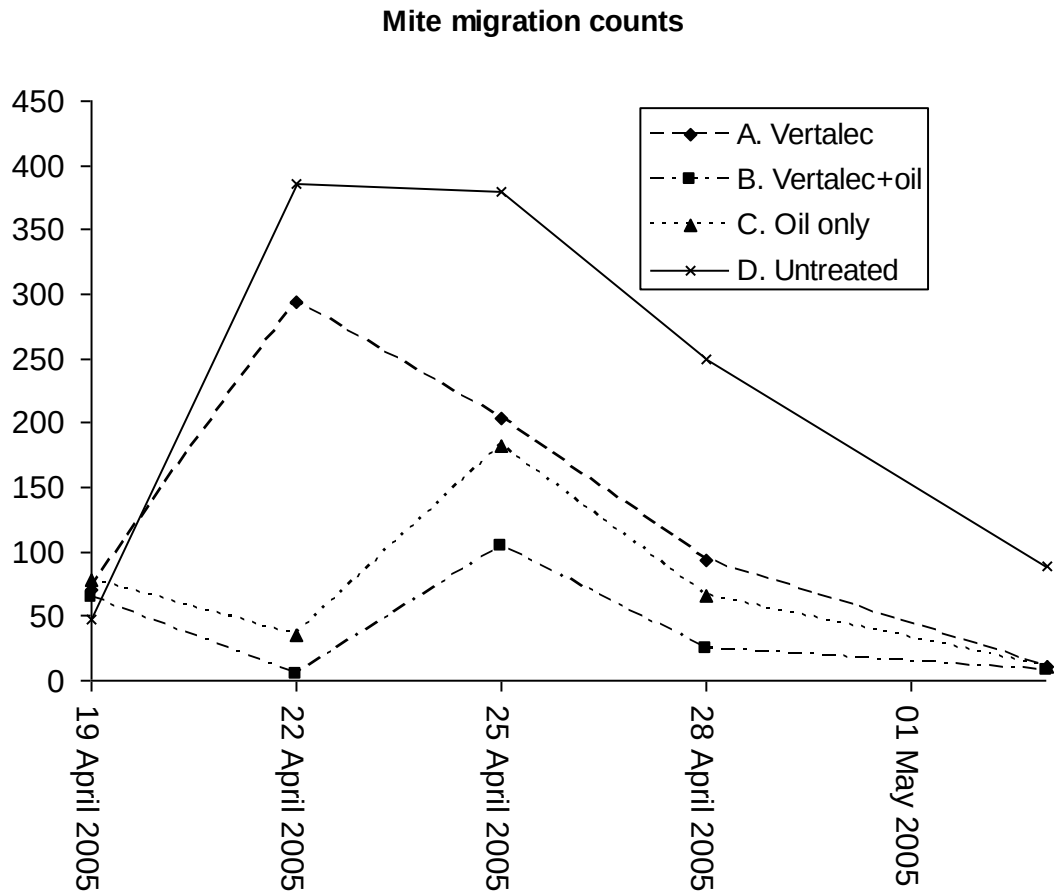


Figure 1. Mean counts of migrated mites on sticky tapes. Treatments were applied on the 19 and 25 April.

All material, including the untreated stems, showed a decline of mites appearing from the galls over time. Although some of the material received a second treatment application (25 April) this did not appear to have an influence on migrating mite numbers as all treatments showed a similar downward trend (Fig. 1). Despite this there was a relatively healthy population of mites in galls of all treatments as discovered from direct observation of the galls at the end of the experiment (by removing the outer leaves, Table 1).

Table 1. Score of mites estimated in galls at end of experiment

Treatment	A	B	C	D
Mean score	1.0	2.0	2.3	1.6
St dev (n=15)	1.3	1.4	1.1	1.4

Score: 0: no mites, 1: 10-20 mites, 2: 20-100 mites, 3: >100 mites

There was no indication that the number of mites was reduced in the Vertalec treated galls, indeed, the Codacide oil treated galls had the highest number of mites remaining in the galls.

The quartered and incubated galls became covered by slime moulds within 6 days of inoculation with the exception of those on the dodine-containing media. Reference SDAY plates on which Vertalec suspension had been spread directly, showed visual signs of growth after 6 days and a reasonable amount of fungal hyphae growth after 3 weeks (Fig. 2). The dodine-containing SDAY plates did not show any sign of fungal growth from Vertalec (after 3 weeks).

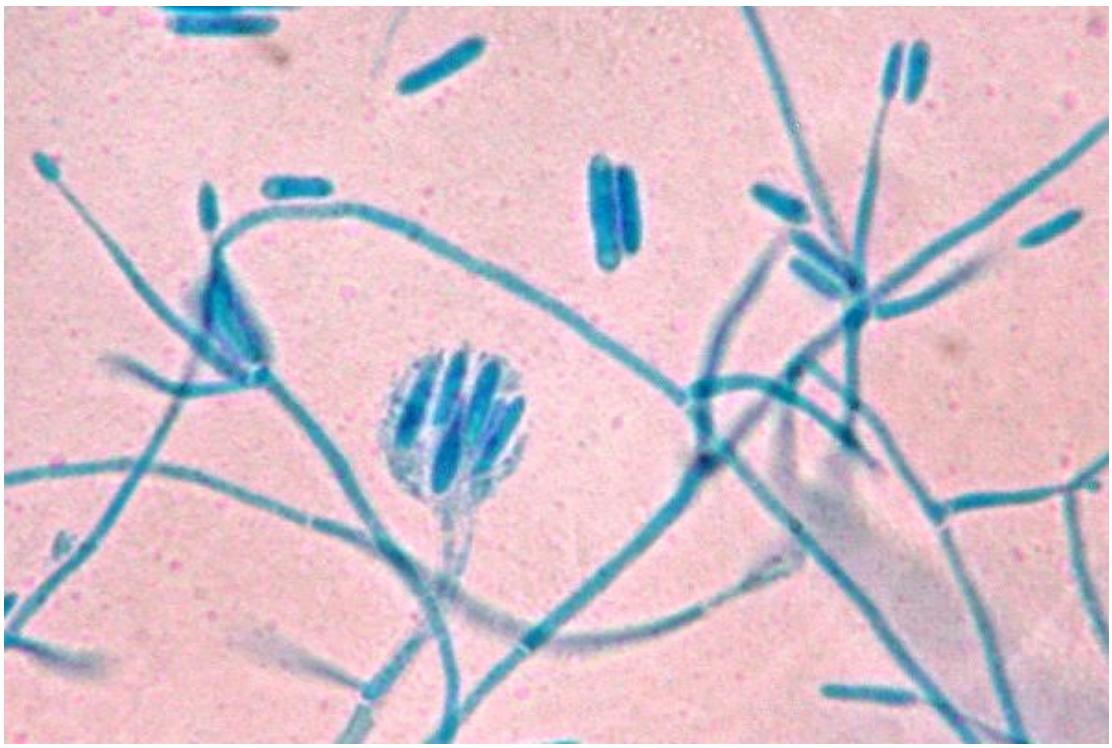


Figure 2. Conidiophores of *Lecanicillium longisporum* grown directly from 'Vertalec' on SDAY media. The uniformly coloured spores of 3-5 µm long, together with the occurrence of conidiophores were used as positive control for *L. longisporum* identification.

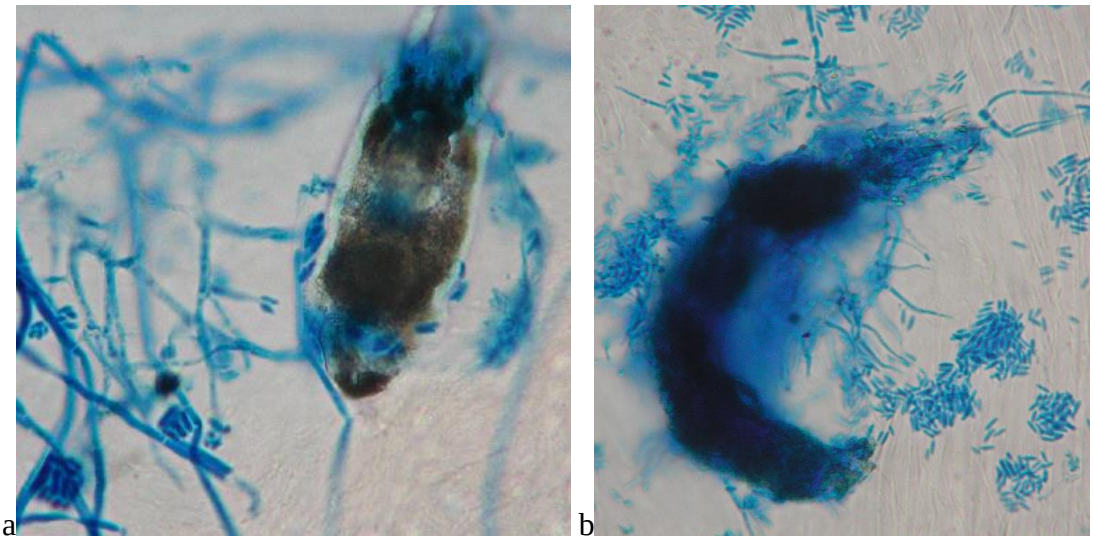
Microscopical examination of the fungus and mites within the buds

In samples directly observed by microscopy for the mites, treatments that included Codacide oil seemed to be prone to fungal infestation (Table 2). Even though the

buds from these treatments contained the highest number of mites by the end of the experiment, all of the buds showed at least some fungal infestation of mites. Possible, but inconclusive (putative), *L. longisporum* was found in both of the treatments containing oil (Fig. 3).

Table 2. Fungal assessment of mites located within the bud.

Treatment	Samples with mites left (out of 15)	Signs of clear fungal infestation	Putative *L. longisporum* infestation
A. Vertalec	6	1	0
B. Vertalec+oil	10	10	5
C. Codacide oil only	14	14	2
D. Untreated	9	3	0



a b

Figure 3. *Lecanicillium longisporum* infected mites from the blackcurrant galls. The fungus appears to be growing in a gelatinous layer on the surface of this partly opaque mite (a) from the Vertalec+oil treatment B. (b) Hyphae appear to be growing around and penetrating the body of this severely degraded mite, (Codacide oil only, treatment C).

Microscopical examination of fungal infested mites on sticky traps

Sticky tapes with migrated mites, mounted on microscope slides in lactophenol stain and incubated for various periods (I. direct examination, II. incubated for 3-4 days,

III. incubated for 6-8 days) to give ample time for spores to infest the migrated mites, showed an increase in fungal growth on the later dates in the experiment, and after prolonged incubation of the sticky tape in a humid chamber.

L. longisporum was not found at the start of the experiment (19 April 2005), but was found associated with mites in all of the treatments, except the untreated, 3 days after the application of the treatments. However, after 8 days of treatment the putative *L. longisporum* was also found in the untreated cultures and Vertalec+oil treatments only. Occurrence of *L. longisporum* was very infrequent with only one or two positive identifications in any treatment at any date.

A further complication in the interpretation of the fungus infection was the ability of aniline blue to stain the mites themselves (sometimes very darkly, Fig. 4). Over time the number of blue-stained mites increased irrespective of treatment (Fig. 5). However, even after careful observation, no morphological differences between stained and unstained mites were detected (Mike Easterbrook, pers.obs.). Mites that were parasitized by fungal hyphae were often stained blue.

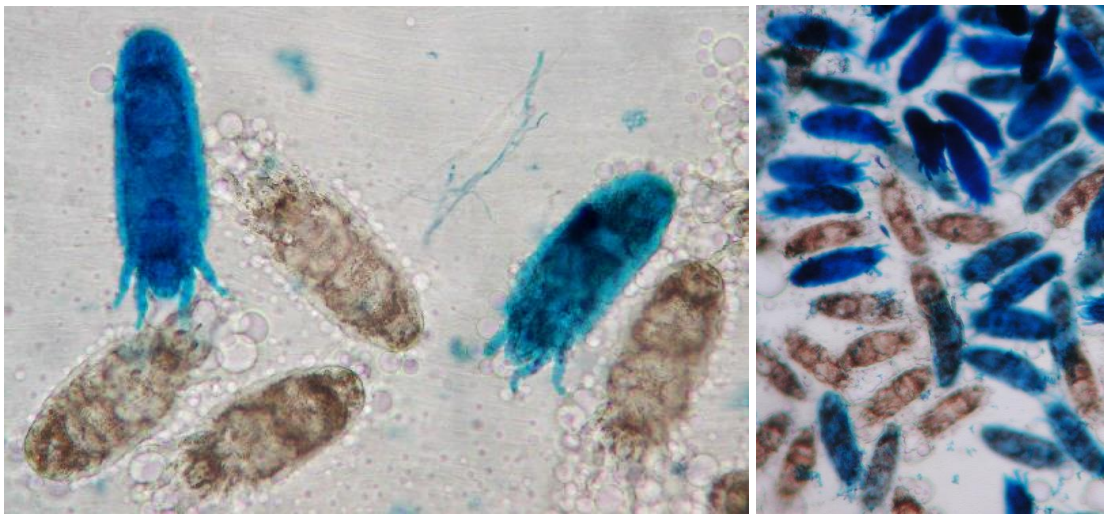


Figure 4. A proportion of the mites stained blue in lactophenol with aniline blue stain. Pictures are from treatment A (Vertalec only).

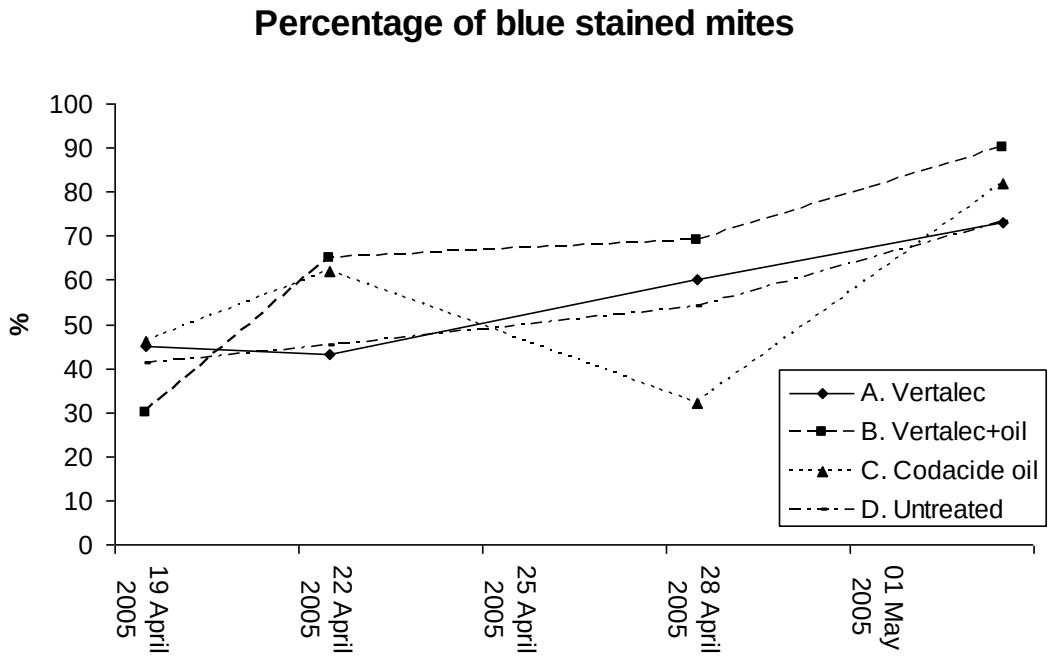


Figure 5. The percentage of mites that stained blue after mounting in lactophenol with 0.05% aniline blue stain.

Discussion

The number of mites migrating up stems from the buds treated with Codacide oil declined over time. This may be due to a 'barrier' effect of the oil preventing mites leaving the galls. This is also supported by the fact that buds treated with the oil had the highest number of mites resident in the buds at the end of the experiment (the proportion of mites alive has not been assessed).

Codacide oil was used to facilitate the fungal spores sticking to the surface of the mites. However this effect was not supported by the data presented here as very few *L. longisporum* parasitation incidences were observed. It was predicted that mites would pick up spores from the blackcurrant stem during migration towards other buds higher on the stem. However, even during incubation for up to 8 days under optimal conditions for fungal growth, putative *L. longisporum* was only detected in one tenth of the samples (treated and untreated stems), indicating the occurrence of indigenous *L. longisporum* related species.

The use of Codacide oil did promote an increased fungal infestation of the mites within the galls, chiefly by *Alternaria* and *Penicillium*-like species. Synergistic effects on spore-germination have not been demonstrated, but it is speculated that the oil provides a nutrient source for the fungus.

Positive identification of *L. longisporum* inside the galls by direct microscopical observation was limited to the Vertalec treatment where Codacide oil had been included as an adjuvant, but also in the adjuvant-only treatment, further supporting the occurrence of indigenous *L. longisporum* related species. As the treatments were applied at a time when buds were partly open, and incubated under fungus-stimulating conditions, it seems likely that other similar species could thrive. However, the very small effect of the treatments overall seem to suggest a very low pathogenicity of the Vertalec strain of *L. longisporum* for the blackcurrant gall mite.

The fungus almost always surrounded the body of the mycotized mites. Only in a few cases was a fungus seen penetrating a mite (not *L. longisporum*), generally via the

mouthparts. It was not possible to establish whether these fungi were the cause of mite fatality or whether the fungus infestation occurred after death.

The blue staining of mycotized mite bodies hampered a thorough investigation of internal mycotization, but in the majority of cases it was possible to determine that mycotization occurred on the surface of the mites, even when mites had been degraded to a ghost-like appearance. This is in contradiction to the work by Kanagaratnam (1981) who isolated seemingly severely pathogenic *Verticillium lecanii (=L. longisporum*) strains from blackcurrant gall mite samples from England, and showed internal growth of hyphae by staining with methylene blue. The latter stain might not have the disadvantage that aniline blue gives (staining of mites), but in our study it only stained the fungi faintly and did not stain *L. longisporum* spores at all.

Direct isolation of fungus from the plant material did not prove worthwhile. The fungus grew slowly on standard SDAY plates, by the time it could clearly been seen (6 days), the plates with plant material were swamped with fungi, particularly slime moulds. The addition of the selective agent dodine at the recommended concentration of 0.45g/l did not allow any growth, even after 3 weeks.

Conclusions

The results of this laboratory experiment suggest that the Vertalec strain of *L. longisporum* is not pathogenic enough to be used as a biocontrol agent against blackcurrant gall mite.

Reference

Kanagaratnam P, Hall RA and Burges HD (1981) Effect of fungi on the black currant gall mite, *Cecidophyopsis ribis*. *Plant Pathology*, **2**, 117-118.