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The impact of two introduced biocontrol agents, *Phytomyza vitalbae* and *Phoma clematidina*, on *Clematis vitalba* in New Zealand

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Abstract

Insecticide and fungicide exclusion experiments were performed to determine the impact of two biological control agents, an agromyzid leaf-mining fly *Phytomyza vitalbae* Kaltenbach and a coelomycete fungal pathogen *Phoma clematidina* (Thüm.) Boerema, on the growth and percentage cover of *Clematis vitalba* L. (Ranunculaceae) plants. Both insecticide and fungicide treatments significantly reduced control agent damage to *C. vitalba* leaves over one growing season at Blenheim, New Zealand. However, damage attributable to both agents was rather low and population peaks of both agents occurred in late fall, after the main period of stem growth. There was no significant impact of treatment on growth and only a minor (8–10%), but significant, reduction in percentage cover of *C. vitalba* was recorded. Disease symptoms were generally only expressed late in the growing season, when leaves were senescent, and were correlated with *Py. vitalbae* damage. Therefore, we tentatively conclude that alone, *Ph. clematidina* is insufficiently pathogenic to induce disease symptoms during the main growing season of *C. vitalba*. Selection criteria for any future potential biocontrol pathogen, therefore, need to evaluate inherent epidemiological factors before introduction, to ensure the candidate agent is an aggressive primary pathogen that can exert maximum disease attack on the target plant. Furthermore, the potential of *Py. vitalbae* to exist as an asymptomatic endophyte indicates that extra care may be required when assessing survey results for non-target attack, and when testing candidate pathogen biological control agents for host specificity.
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1. Introduction

Evaluation is arguably the most important phase of biological control because it provides valuable data for decision-making within a particular biological control project, and also contributes generally to ecological theory on biological control and plant–herbivore interactions (Briese, 2004). However, evaluation is not always undertaken to the extent necessary, largely because of a funding-driven emphasis on the finding, testing, and delivery of agents (Briese, 2004). For example, Dhileepan (2003) observed that quantitative data were available for only 38% of target weeds in Australia at an individual plant level and 20% at a plant population level. The corresponding figures for New Zealand are similar (Dr. K. Potter, unpublished data).

Recent concerns about the potential non-target impacts of biological control (e.g., Louda et al., 2003) are resulting in tighter controls over the importation and release of biological control agents worldwide (Sheppard et al., 2003). In New Zealand, the Hazardous Substances and New Organisms (HSNO) Act (1996) requires a rigorous risk analysis to support the potential introduction of biological control agents that not only considers the potential of each proposed agent to attack non-target plant species, but also

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assesses their likely contribution to the biological control of the target weeds (Fowler et al., 2000). Evaluating weed biological control programs is therefore essential to improve our ability to predict the impact and safety of future introductions and, therefore, underpins the continued use of biological control as a tool against invasive alien weeds.

Old man's beard, *Clematis vitalba* L. (Ranunculaceae), a vine that is native to Europe, and extends east to the Caucasus, was introduced to New Zealand as an ornamental before 1920 (Hill et al., 2001). It is now widespread, threatening the existence of many New Zealand native forest remnants (Bungard et al., 1997). Old man's beard kills native trees and shrubs by smothering the canopy with dense foliage which reduces light levels and weighs down and collapses the branches of host trees (Hume et al., 1995).

Two biological control agents, an agromyzid fly *Phytomyza vitalbae* Kaltenbach, and a coelomycete fungal pathogen *Phoma clematidina* (Thüm.) Boerema, were released against *C. vitalba* in 1996 (Gourlay and Wittenberg, 2000). The larvae of *Py. vitalbae* mine *C. vitalba* leaves, reducing photosynthetic area and inducing leaf senescence (Hill et al., 2001), whereas *Ph. clematidina* infection was recorded to cause leaf spotting and vine wilting (Spiers, 1995). Both agents established rapidly and spread (Hill et al., 2001) and the speed with which *Ph. clematidina* dispersed within New Zealand and the co-occurrence of the two agents at new sites raised the possibility that the two agents were synergistic in their effects on *C. vitalba* leaves (Hill et al., 2004). However, experimental work by Hill et al. (2004) indicated that *Py. vitalbae* is unlikely to be a good vector of *Ph. clematidina*, and that feeding damage by adult flies did not enhance fungal establishment. Nevertheless, Hill et al. (2004) noted that before *Ph. clematidina* was introduced, *Py. vitalbae* leaf mines were usually brown, yet following establishment of the fungus leaf mines were usually black. Although the cause of the discoloration was never formally identified, it was suspected that the *Ph. clematidina* was invading larval leaf mines.

Surveys to investigate potential non-target impacts of *Py. vitalbae* and *Ph. clematidina* are ongoing, although preliminary results have been published for *Py. vitalbae* (Paynter et al., 2004). This experiment was, therefore, designed to (a) quantify the impact of *Py. vitalbae* and *Ph. clematidina*, both separately and in combination on *C. vitalba* growth, and (b) determine whether there is any evidence for competition or synergy between *Py. vitalbae* and *Ph. clematidina*.

2. Materials and methods

2.1. Field site

Clematis vitalba vines were located near Blenheim in a wasteland area alongside the Wairau River (41°52′E, 173°73′N) in the South Island of New Zealand. Semi-deciduous *C. vitalba* vines were growing under a largely exotic partial canopy of poplar (*Populus*) and wattle (*Acacia*) trees. This site was selected due to the presence of an abundance of discretely growing vines, to which treatments could be allocated, and because both biological control agents were known to be well established. Forty discrete mature *C. vitalba* vines were selected and randomly assigned to one of four treatments (10 vines per treatment), which were applied every 6 weeks from 15 September 2003 until 22 April 2004 as follows:

1. A control treatment: water only, sprayed to run off.
2. A fungicide treatment: Carbendazim (methyl-2-benzimidazol carbamate); 1 g each of BavistinDF (BASF New Zealand, Auckland, New Zealand) and Captan (Nufarm, Auckland, New Zealand) mixed with 1 L of water and sprayed to run off.
3. An insecticide: treatment 200 g/L azinphos-methyl (Gusathion, Bayer New Zealand, Auckland, New Zealand)—1 g/L sprayed to run off.
4. A combined insecticide plus fungicide treatment: plants initially treated with fungicide, according to treatment 2, then allowed to dry before being treated with insecticide, as for treatment 3.

Work was also conducted at a second field site at the Mangawharariki River, near Mangaweka (39°48′S, 175°49′E) in the North Island of New Zealand. However, this field site was destroyed by floods and subsequent landslides in February 2004.

2.2. Data collection

2.2.1. Stem growth

Six randomly selected shoots on each vine were marked on 15 September 2003 and were subsequently measured (including lateral growth if lateral shoots appeared) every 6 weeks from 5 November 2003 until 8 March 2004. Difficulty in relocating many of the shoots originally tagged on 15 September resulted in new shoots being tagged on March 8 and measured until 3 June.

2.2.2. Percentage cover

A 50×50 cm permanent quadrat was set up for each vine. On 27 January, 22 April, and 3 June 2004, a close-up photograph of the foliage growing within each quadrat was taken for analysis of percentage cover of *C. vitalba* and incidence of fungal and insect damage. This was done using Digital Sampling Method, Version 1.00 (Landcare Research, New Zealand) as follows: 100 points on each photograph were randomly generated and each was scored, according to whether it scored positive for *C. vitalba* vegetation (and if the control agents were present or absent), competing vegetation or litter. *C. vitalba* percentage cover was then calculated as the sum of randomly generated points that scored positive for *C. vitalba* vegetation. In addition, on 22 April 2004, the degree to which each vine was shaded by the canopy was estimated by photographing the canopy directly above each adult vine and using Digital Sampling Method to estimate 'canopy gap', defined as the proportion of the photograph that contained clear sky.

2.2.3. Identification of agents

All leaf mines were assumed to be *Py. vitalbae*. Concurrent studies revealed that the only other species to mine *C. vitalba* leaves in New Zealand is the native leaf-mining fly *Phytomyza clematidi* Watt, which feeds on native New Zealand *Clematis* species and very rarely attacks *C. vitalba* (<0.25% of mines examined, Q. Paynter; unpublished data).

Although *Ph. clematidina* was reputedly present in New Zealand before the biological control program against *C. vitalba* (Smith and Cole, 1991), all isolates of *Ph. clematidina* were assumed to be the isolate deliberately released against *C. vitalba* in New Zealand. Although the isolates cannot be distinguished morphologically, the isolate released against *C. vitalba* was much more pathogenic to *C. vitalba* (Spiers, 1995, 1996) than the isolates recorded previously in New Zealand (Smith and Cole, 1991), and conspicuous damage to *C. vitalba* plants was recorded soon after its release (Hill et al., 2001).

Leaves displaying necrosis, causing blackish patches (see Hill et al., 2004), were recorded as having symptoms of fungal attack. To differentiate between leaf rot caused by *Ph. clematidina* and that caused by other fungal pathogens, identification of *Ph. clematidina* was undertaken by microbiological isolation methods. In September 2003 and January and June 2004, samples of foliage exhibiting symptoms of fungal attack were collected from each plant. Smaller sub-samples (i.e., not all plants were sampled) were collected in November and December 2003. Leaves, leaf petioles, or stems were placed in paper bags, kept cool, and taken to the laboratory. Material that displayed no obvious disease symptoms was collected from a minimum of one plant per treatment at each sample date, to examine the possible 'endophytic phase' of fungi present on the plant. Plant materials were examined within 5 days of collection. In the laboratory, isolations of fungi were attempted from a range of lesions selected to represent the diversity of symptoms from each sample. Small pieces of tissue (ca. 3 mm^2) were cut from the edge of diseased areas and surface sterilized by immersion in 2% hypochlorite for 1–3 min, followed by rinsing twice in sterile water. The tissue fragments were blotted dry with sterile filter paper and placed in 9-cm petri dishes containing potato dextrose agar (Difco Labs, Detroit, MI, USA) with 0.02% streptomycin sulphate (Sigma, St. Louis, MI, USA). Plates were incubated under near ultraviolet and white light (12 h photoperiod) at temperatures of 22 ± 2 °C (day) and 18 ± 2 °C (night). Tissue fragments from asymptomatic leaves were processed in an identical manner.

2.3. Analysis

A repeated measures analysis of covariance was performed using GENSTAT 6.2 (VSN International, Hemel Hempstead, UK) to determine the effect of sample date (henceforth 'time', corresponding to three sample dates: 27 January, 22 April, and 3 June 2004) and treatment (control, insecticide, fungicide, and insecticide plus fungicide) on the growth of the vines (cumulative growth averaged for the six stems per vine recorded at each date). Similar analyses were performed to determine the effect of treatment on percentage cover of *C. vitalba*, the proportion of leaves mined by *Py. vitalbae* or showing symptoms of pathogen attack, and the proportion of leaf area damaged by each biological control agent. An angular transformation was performed on all proportion data before analysis. Growth data were $\log(n+1)$ transformed before analysis. For all the above analyses, 'canopy gap' was included as a covariate because the degree to which each vine was shaded was not uniform and Baars and Kelly (1996) demonstrated that while *C. vitalba* has a high degree of tolerance to shading, like other weedy vines, it is characterized by rapid growth in high-light environments. Finally, the effect of treatment on the proportion of plants from which *Ph. clematidina* was isolated was analyzed. For this analysis, binomial errors were specified (see Crawley, 1993).

Only data from the Blenheim field site were analyzed, because the Mangaweka field site was destroyed by floods shortly after the January census.

3. Results

3.1. Growth

There was a significant positive correlation between the extent of canopy gap and stem growth ($F_{1,34} = 6.61$, $P < 0.05$), indicating that shading reduced stem growth. Treatment, however, had no significant effect on stem growth ($F_{3,34} = 0.33$, n.s.).

3.2. Percentage cover of C. vitalba

Percentage cover of *C. vitalba* was significantly correlated to the extent of canopy gap and varied significantly over time (Table 1). Treatment also had a minor but statistically significant effect on percentage cover of *C. vitalba*, which was lower (ca. 75%) in control quadrats compared to those treated with insecticide and/or fungicide (ca. 83–85%; Fig. 1).

Table 1
Repeated measures analysis of variance for *C. vitalba* percentage cover, estimated from photographs of permanent quadrats taken in January, April, and June 2004

Source of variation	Sum of squares	*df*	Variance ratio
Subject stratum			
Treatment	0.078	3	4.50**
Canopy gap	0.286	1	16.60***
Subject · time stratum			
Time	0.077	2	8.49***
Time · treatment	0.015	6	1.62
Residual	0.009	71	
Total	118	1.9	
		78	

** $P < 0.01$.
*** $P < 0.001$.

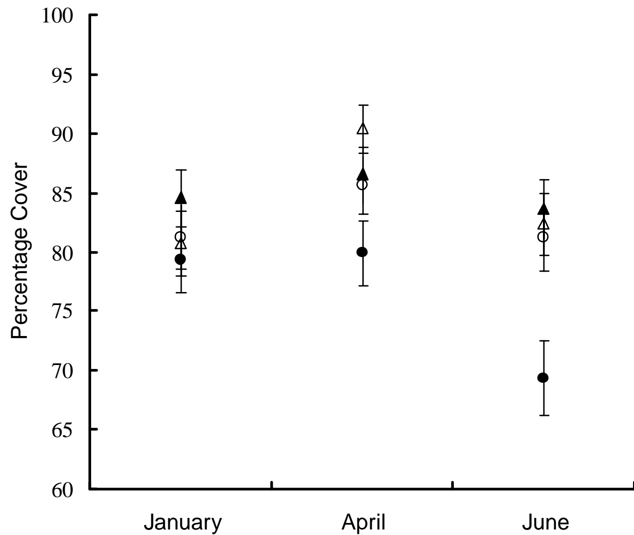

Fig. 1. Impact of treatment on the mean (±SE) percentage cover of *C. vitalba*, calculated by multiplying the back-transformed parameter estimates (±SE) by 100. (●) Control treatment; (▲) fungicide treatment; (○) insecticide treatment; and (△) combined (fungicide + insecticide) treatment.

3.3. Damage due to Py. vitalbae

The proportion of leaves mined by *Py. vitalbae* was not affected by canopy gap ($F_{1,35}$=1.41, n.s.). However, both treatment ($F_{3,35}$=15.98, P<0.001) and time ($F_{2,71}$=40.67, P<0.001) and their interaction term ($F_{6,71}$=3.73, P<0.01) were significant, indicating the treatment effect varied through time. Mines began to develop soon after leaves appeared, however, treatment had no significant effect on the proportion of mined leaves recorded in January, when very few leaves were mined. However, by June, 20–25% of leaves in the control and fungicide treatments were mined, while the proportion of mined leaves remained low in the insecticide and insecticide plus fungicide treatments (Fig. 2A), indicating insecticide reduced the abundance of *Py. vitalbae* by 75–90%.

Estimates of the percentage leaf area destroyed by mines followed a similar pattern (Fig. 2B). However, because individual leaf mines only destroyed a fraction of each leaf, the percentage of leaf area destroyed by *Py. vitalbae* was estimated to be rather lower than the proportion of mined leaves (up to a maximum of ca. 5% of total leaf area in the control plots recorded in June; Fig. 2B).

3.4. Damage due to Ph. clematidina

Although there was a trend for a greater proportion of leaves with symptoms of pathogen attack in the control plants compared with the treated plants, the proportion of leaves with symptoms of fungal attack estimated from the digital photographs did not vary significantly according to treatment ($F_{3,35}$ = 2.54, P ~ 0.07; Fig. 3A). Canopy gap was not significant either ($F_{1,35}$ = 0.19, n.s.). The proportion of visibly infected leaves did vary with time: the first symptoms of fungal attack appeared soon after leaves formed,

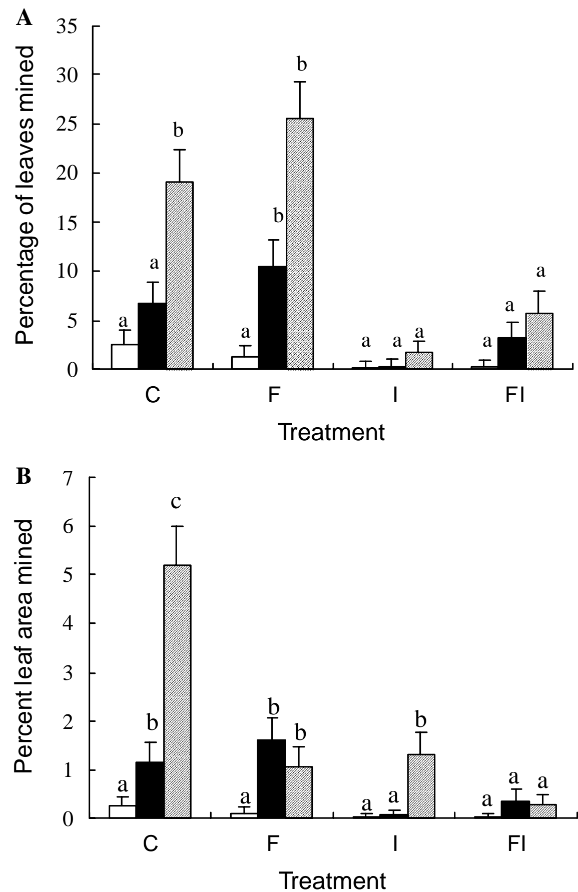

Fig. 2. (A) Impact of treatment on percentage of leaves mined (±SE); (B) % leaf area mined (±SE) recorded on 27 January (no fill), 22 April (black fill), and 3 June 2004 (diagonal bars). *X*-axis abbreviations: C, control; F, fungicide treatment; I, insecticide treatment, and FI, combined (fungicide+insecticide) treatment. Different letters denote significant differences between treatments for each sampling date.

but few leaves showed symptoms of attack in January, compared to later in the year ($F_{2,71}$ = 13.97, P < 0.001). In contrast, examination of fungal isolates from leaf samples revealed treatment significantly affected the proportion of plants from which *Ph. clematidina* was isolated in January and June 2004 (χ^2 = 10.464, *df* 3, P < 0.05 and χ^2 = 18.22, *df* 3, P < 0.001 for January and June, respectively; Fig. 3B), but not in November 2003 (χ^2 = 1.22, *df* 3 n.s.). In September, *Ph. clematidina* was isolated from two out of four samples that showed no external symptoms of disease. In November, January and June, respectively, *Ph. clematidina* was isolated from two out of two, none out of five and three out of four leaf samples lacking external symptoms of disease.

Estimates of the percentage leaf area destroyed by fungus indicated treatment had a significant effect ($F_{3,35}$ = 3.12, P < 0.05). Fungal symptoms were significantly reduced by the insecticide and the combined fungicide plus insecticide treatments, but not by the fungicide treatment (Fig. 3C). Time ($F_{2,71}$ = 18.27, P < 0.001) and the interaction term between treatment and time ($F_{6,71}$ = 4.26, P = 0.001) were also significant, indicating the treatment effect varied through time. As for *Py. vitalbae*, the percentage of leaf

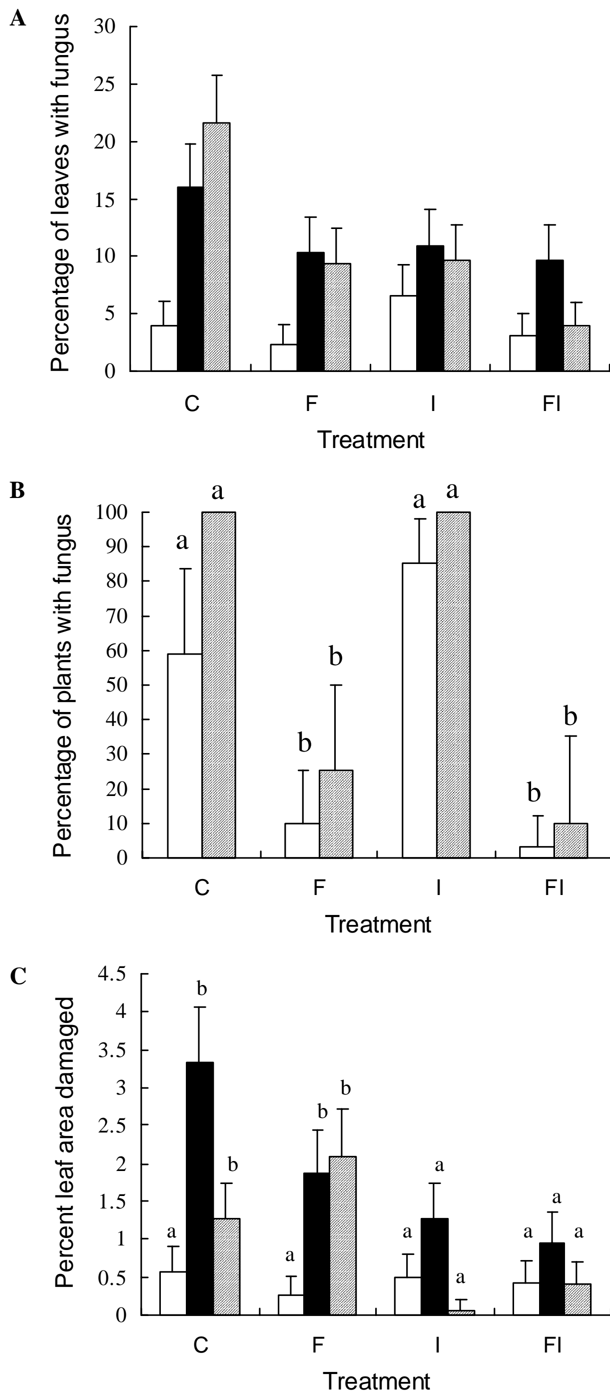

Fig. 3. (A) Impact of treatment on percentage of leaves with fungal symptoms (±SE); (B) proportion of plants from which *Ph. clematidina* was isolated (±SE); (C) % leaf area damaged (±SE) recorded on 27 January (no fill), 22 April (black fill), and 3 June 2004 (diagonal bars) (graphs a and c; January and June 2004 only, graph b). *X*-axis abbreviations as for Fig. 2. Different letters denote significant differences between treatments for each sampling date.

area destroyed by fungus was rather low (reaching an average of just 3.3% in the control plots in April; Fig. 3C).

Although cultural isolation confirmed *Ph. clematidina* as the primary cause of the disease symptoms observed on the host (overall approximately 51% of all isolates were *Ph. clematidina*), there were some occasions, where fungal damage could not be attributed to *Ph. clematidina* alone. Isolation of other leaf pathogens from diseased tissues where *Ph. clematidina* was not isolated included; *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc., *Phomopsis* sp., and several species of *Fusarium*, all of which are previously recorded as primary leaf pathogens in New Zealand (Waipara et al., 2005). Additional fungi isolated in association with disease symptoms [*Alternaria alternate* Fries (Keissler), *Epicoccum nigrum* Link, *Gliocladium roseum* Bainier, and assorted Mucorales species] were identified as secondary saprophytes. Preparations of the *Ph. clematidina* isolates have been placed in the International Collection of Micro-organisms from Plants (ICMP) as vouchers (*Phoma clematidina* ICMP 15898 and *Phoma clematidina* ICMP 15899).

3.5. Damage from both agents

Estimates of the percentage leaf area destroyed by both agents combined again indicated treatment ($F_{3,35} = 10.27$, $P < 0.001$), time ($F_{2,71} = 27.53$, $P < 0.001$), and their interaction term ($F_{6,71} = 3.12$, $P = 0.01$), were significant. For April and June, the insecticide treatment reduced damage significantly more than the fungicide treatment (Fig. 4). Moreover, compared with the insecticide alone treatment, there was no additional reduction in damage in the fungicide plus insecticide treatment, indicating insect herbivory, mainly by *Py. vitalba*, caused the most damage to *C. vitalba* leaves.

Regressions of the proportion of leaves with fungal symptoms versus the proportion of leaves that were mined by *Py. vitalbae* indicated there was no significant relationship between the abundances of both agents in January and April 2004 (Figs. 5A and B). However, in June 2004 there was a highly significant positive correlation between the two agents (Fig. 5C). Respectively, 18.4, 13.8, and 9.8% of

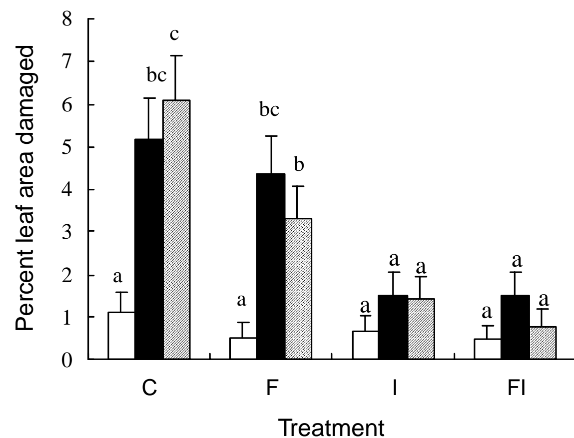

Fig. 4. Impact of treatment on the percentage of leaf area damaged by both species (±SE) recorded on 27 January (no fill), 22 April (black fill), and 3 June 2004 (diagonal bars). *X*-axis abbreviations as for Fig. 2. Different letters denote significant differences between treatments for each sampling date.

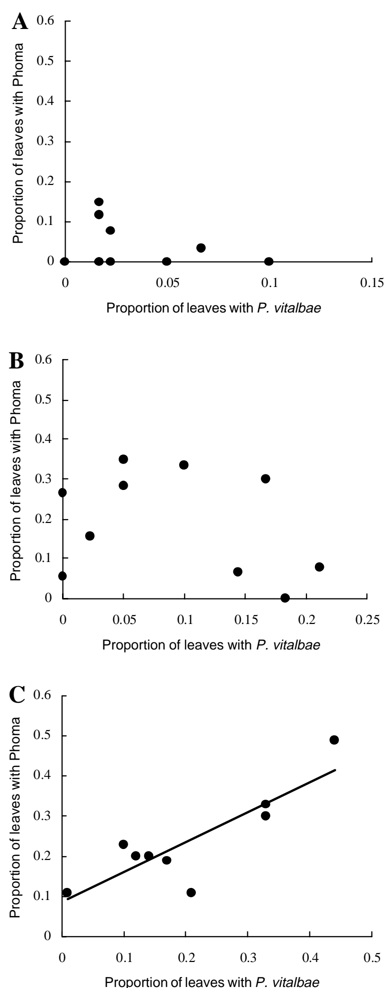

Fig. 5. Correlations between agent abundances (A) January Number of leaves with *Ph. clematidina* symptoms, $r^2 = 0.0661$, n.s., (B) April Number of leaves with *Ph. clematidina* symptoms, $r^2 = 0.1077$, n.s., and (C) June Number of leaves with *Ph. clematidina* symptoms = 0.7489 (Number of leaves with *Py. vitalbae* mines) + 0.0861, $r^2 = 0.7304$, $P < 0.01$.

leaves were attacked by *Py. vitalbae* alone, *Ph. clematidina* alone, and both agents combined in June. Analysis of the count data indicated that the number of leaves attacked by both agents was higher than would be expected if the distribution of agent attack was random with respect to one another ($\chi^2 = 14.8$, $P < 0.01$).

3.6. Representative damage?

Data from the January 2004 census at Mangaweka and from surveys conducted at 12 sites throughout New Zealand in 2005 enabled us to determine whether the damage attributed to *Py. vitalbae* at Blenheim was representative of levels throughout the country. *Py. vitalbae* was present at all sites, but the level of attack was rather variable with the proportion of mined leaves ranging from 6.4 to 96% (Table 2). The levels of *Py. vitalbae* recorded in the current study (up to 25% of leaves mined) were, however, somewhat lower than the average of 47% (Table 2).

Table 2
Proportion of *C. vitalba* leaves mined by *Py. vitalbae* recorded at 12 sites throughout New Zealand, in February and March 2005 and at Mangaweka in January 2004

Location	Number of leaves sampled	Proportion of leaves mined
Rotorua, 38°08′S, 176°14′E	64	0.17
Te Wairoa, 38°13′S, 176°21′E	54	0.52
Taihape, 39°41′S, 175°47′E	140	0.48
Mangaweka, 39°48′S, 175°49′E	610	0.06
Bledisloe Park, Palmerston North, 40°23′S, 175°37′E	186	0.48
Summit Rd, Banks Peninsula, 43°40′S, 172°37′E	42	0.95
Gebbies Pass, Banks Peninsula, 43°41′S, 172°38′E	48	0.96
Western Valley Rd, Banks Peninsula, 43°45′S, 172°48′E	35	0.89
Kinloch Rd, Banks Peninsula, 43°47′S, 172°91′E	36	0.19
Peel Forest, 43°54′S, 171°15′E	84	0.92
Dunedin Botanical Gardens, 45°51′S, 170°31′E	52	0.15
North Dunedin, 45°52′S, 170°31′E	18	0.11
Colinswood, Dunedin, 45°52′S, 170°35′E	33	0.18
Average		0.47

At each site, at least one *C. vitalba* stem was cut 100 cm from the tip and the total number of leaves and the number of leaves with *Py. vitalbae* mines were counted.

Similar surveys to determine the relative abundance of *Ph. clematidina* have not yet been conducted. The level of *Ph. clematidina* attack recorded on control plants at Blenheim in January 2004 (3.94% of leaves with fungal symptoms) was somewhat lower than that recorded on control plants at Mangaweka in January 2004 (14.28% of leaves with fungal symptoms).

4. Discussion

Even though the treatments significantly reduced or excluded both biological control agent species, which were common on non-treated plants (e.g., in June 2004, up to 25% of leaves were mined by *Py. vitalbae*, and *Ph. clematidina* was isolated from all plants that had not been treated with fungicide; Figs. 2A and 3B, respectively), only shading (canopy gap) significantly affected *C. vitalba* growth (see Section 2.3 above). Both biocontrol agents damaged only a small fraction of the total leaf area and most damage from both agents occurred late in the season, after the main period of stem growth. Nevertheless, a minor (8–10%) but significant reduction in *C. vitalba* percentage cover was recorded in the control treatment, compared with both the fungicide and the insecticide treatments. If *C. vitalba* dominates to such an extent that light availability is a limiting resource for competing species, then models of plant competition predict that any reduction in a plant's ability to sequester that limiting resource may promote coexistence of species (Grover, 1994). Furthermore, the impacts of both

Ph. clematidina and *Py. vitalbae* may have been underestimated slightly because neither the insecticide nor the fungicide treatments excluded them completely. Moreover, the abundance of *Py. vitalbae* was much greater at a number of other sites surveyed (Table 2), indicating that this agent may have a greater impact at other sites. Nevertheless, it seems likely that the modest levels of defoliation attributed to *Ph. clematidina* and *Py. vitalbae* in this study had, at best, only a minor impact on *C. vitalba* competitiveness.

It is not yet clear why the impact of *Ph. clematidina* and *Py. vitalbae* was so limited. Three species of parasitoids have been reared from *Py. vitalbae* leaf mines in New Zealand (Hill et al., 2001) and they may limit the abundance of this species. Furthermore, although many plant pathogens with weed biocontrol potential have been described, most lack sufficient aggressiveness to overcome plant defenses (Evans et al., 2001). Additionally, many of these pathogens have a latent phase in their life cycles, i.e., they are dormant, not causing any significant systemic disease until latency is terminated by cofactors, such as environmental stresses impacting on the host or leaf senescence (Agrios, 1998). Latent pathogens can exist in a host as asymptomatic endophytes, which is possibly what was observed in our survey. Even though the introduced strain of *Ph. clematidina* was selected for its pathogenicity, it may nevertheless lack the ability to exert early-season disease pressure on the weed, or a latent phase is a normal part of its life cycle on *C. vitalba*.

Isolation of other leaf pathogens from diseased tissues confirms *C. vitalba* is susceptible to other plant diseases present in New Zealand. Interactions between such pathogens and *Ph. clematidina* may also be a factor contributing to the relatively low damage levels attributed to *Ph. clematidina* in the present study, as a complex of plant pathogens on the same host can be both synergistic or inhibitory to disease expression (Guske et al., 2004; Morin et al., 1993). Further investigation would be needed to confirm this.

Past experimental evidence indicated that *Py. vitalbae* is not a good vector of *Ph. clematidina* (Hill et al., 2004). However, the significant positive correlation between the proportion of leaves with *Ph. clematidina* symptoms and the proportion of leaves mined by *Py. vitalbae* (Fig. 5C) does suggest that *Py. vitalbae* promoted the entry and colonization by *Ph. clematidina*. This hypothesis is further supported by the finding that the number of leaves attacked by both agents was higher than would be expected if the distribution of agent attack was random with respect to each other. Nevertheless, we cannot rule out the possibility that the fungus and insect are selecting, independently, a host with similar characteristics. However, in June 2004, *Ph. clematidina* was isolated from all insecticide-treated plants, but only a low proportion of fungicide-treated plants (Fig. 3B), yet the proportion of leaves with *Ph. clematidina* symptoms was similar in insecticide-treated and fungicide-treated plants (Fig. 3A). Perhaps *Py. vitalbae* larval mining induced symptoms of disease and although the fungus was ubiquitous in the insecticide-treated plants, it generally remained asymptomatic because there were few *Py. vitalbae* mines present. This interpretation is further supported by the observation that while the proportion of mined leaves was similar in the fungicide-treated and the control plants in June 2004 (Fig. 2A), the leaf area destroyed by mines in the control plants was estimated to be about five times higher than for the fungicide treatment (Fig. 2B). This indicates that the fungicide treatment may have reduced *Ph. clematidina* damage within *Py. vitalbae* mines.

There are numerous examples, where endophytic micro-organsims have been reported to benefit their plant hosts by reducing insect attack (e.g., Azevedo et al., 2000; see Webber, 1981) and by inhibiting both infection and disease progression of many fungal pathogens (Chanway, 1998; Narisawa et al., 2002; Shimizu et al., 2000). This poses the question as to whether, as an endophyte, *Ph. clematidina* could have negative impacts on other biocontrol agents on the same host, such as the sawfly, *Monophadnus spinolae* Klug., which was first released in 1998 (Gourlay and Wittenberg, 2000) and is yet to establish in New Zealand. However, the vast majority of endophytes, especially horizontally transmitted ones commonly found in woody plants, apparently have little or no effect on herbivores (Faeth and Fagan, 2002). Indeed, the incidence of the oak leaf miner *Cameraria* sp., was similarly positively associated with the incidence of endophytes (Faeth and Hammon, 1997a), which had no antagonistic effect on the leaf miner (Faeth and Hammon, 1997b).

Phoma clematidina has been described as a wound pathogen (Smith and Cole, 1991). Our data on the seasonality of disease symptoms and their correlation with *Py. vitalbae* damage lead us to conclude tentatively that *Ph. clematidina* alone is insufficiently pathogenic to induce disease symptoms during the main growing season of *C. vitalba*. Symptoms were generally only expressed late in the growing season, when leaves were senescent or damaged by *Py. vitalbae*. In the disease triangle interaction between plant host, pathogen, and environment, many plant diseases require cofactors such as abiotic stress or specific environmental triggers before progression to a systemic infection and symptoms being expressed on the host (Agrios, 1998). In addition there can also be a natural delay for some plant diseases to become symptomatic, post infection, due to the elicitation of host defence mechanisms, during this time a pathogen can remain present in host tissues as an asymptomatic endophyte until host defences are overcome and symptoms are expressed (Agrios, 1998). It is apparent from our study that the disease cycle of *Ph. clematidina* on *C. vitalba* is significantly affected by such cofactors and interactions for disease expression. The authors are unaware of any other fungal biological control agent that appears to exist primarily as an asymptomatic endophyte. Selection criteria for any future potential biocontrol pathogen, therefore, need to consider and evaluate inherent epidemiological factors before introduction, to ensure the candidate agent is an aggressive primary pathogen that can exert maximum disease attack on the target plant. Furthermore,

the potential for pathogens to exist as asymptomatic endophytes indicates it may not be sufficient to isolate fungi solely from plants showing symptoms of damage when assessing both host-specificity testing or undertaking surveys for non-target attack.

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