Prevalence of Harpellales from Chironomidae larvae in phytotelmata from Punta Lara Forest, Argentina

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Abstract Harpellales (Zygomycota: Trichomycetes) fungi are cosmopolitan obligate inhabitants of the gut of immature insects. A bi-weekly survey of gut fungi associated with chironomid (Chironomidae: Diptera) larvae living in the impounded water from *Eryngium cabrerae* (Apiaceae) phytotelmata from Punta Lara forest, Argentina, was done between Jan 2003 and Dec 2004. Two species of Harpellales were associated with chironomid larvae: *Smittium phytotelmatum* in the hindgut of *Polypedilum* sp. and *Stachylina lentica* in the midgut of both *Polypedilum* sp. and *Metriocnemus eringiotelmatus*. There were no statistically significant differences in the prevalence of these Harpellales between seasons. Various environmental variables (temperature, rainfall, and relative humidity), impounded water volume, pH, and chironomid larval density did not have an effect on the prevalence of the Trichomycetes.

Key words: Chironomidae, Diptera, phytotelmata, Harpellales, Trichomycetes

INTRODUCTION

Trichomycetes are a cosmopolitan endosymbiotic fungal group living in the digestive tract of arthropod hosts. Most trichomycetes are associated with immature stages of aquatic hosts, with relatively few species having been reported in terrestrial arthropods. The relationship between these fungi and their hosts may be either commensalistic, mutualistic or in same cases deleterious (Horn and Lichtwardt 1981, Labeyrie et al 1996, McCreadie et al 2005). Trichomycetes includes two fungal orders, Harpellales and Asellariales. The other two former orders of Trichomycetes, Eccrinales and Amoebidiales, are now know to be protozoans (Cafaro 2005). The Order Harpellales is the most commonly studied, infecting the guts of different aquatic insect larvae (Lichtwardt et al 2001). Recently, the two fungal orders of Trichomycetes have been placed within the subphylum Kickxellomycotina with the suggestion that the term Trichomycetes should be used as 'trichomycetes' to refer to them as ecological group (Hibbett et al 2007).

Chironomids (Diptera: Chironomidae) are known as non-biting midges and their larvae are often a major component of macroinvertebrates in freshwater environments. Chironomid larvae, with a cosmopolitan distribution, are one of the most common hosts of Harpellales, with close to 90 fungal species having been reported from the guts of midges. These trichomycete species are included in the families Legeriomycetaceae and Harpellaceae. The first family includes species associated with the hindgut, whereas the second is represented by species attached to the peritrophic matrix lining the midgut.

Phytotelmata are structures found in terrestrial plants that impound water, such as modified leaves, leaf axils, flowers, stem holes or depressions, open fruit and fallen leaves (Fish 1983). *Eryngium* L. (Apiaceae) is a widespread genus of perennial, rhizomatous herb including some species that capture and retain water in their leaf axils

(Cabrera 1965). The captured water provides a suitable habitat for the development of numerous chironomid larvae and other Diptera (Campos and Lounibos 1999, Donato and Paggi 2005).

Much attention has been given to trichomycete systematics, but only a few ecological investigations have been conducted. Only two studies of Harpellales infecting phytotelmata-inhabiting insects exist, one by Lichtwardt (1994) in Costa Rica and the other by Reeves (2004) in USA. The objectives of the present study were: *i*) to identify the Harpellales fungi associated with midges from phytotelmata in Punta Lara forest, Buenos Aires, Argentina, *ii*) to determine the prevalence and seasonality of the fungi, and *iii*) to determine if the environmental variables (temperature, rainfall, and relative humidity [RH]), impounded water volume, pH or chironomid larval density affected the prevalence of Harpellales.

MATERIAL AND METHODS

Study site and host collections.--

The sampling area (500 m^2) was within the "Integral Natural Reserve" at Punta Lara (34° 51′ 53′′ S, 57° 52′ 23′′ W), Buenos Aires province, Argentina, which is the world′s most austral relict of a "gallery forest" with subtropical vegetation. The study was conducted near a canal in an open field adjacent to the forest. The canal crosses the forest and connects to the Río de La Plata 3 km away. *Eryngium cabrerae* Pontiroli (FIG. 1) grows in densities of about 10 (range 6--12) plants per square meter at the site.

For each sample date (N = 52), one random 20 m long transect was selected within which plants of *E. cabrerae* were chosen every 2 m, until ten plants containing water were available. In addition, another three plants were selected and the same three plants were to be sampled repeatedly throughout the rest of our survey. In this paper, we use "randomly selected plants" for the ten randomly chosen plants, and "selected plants"

when we refer to the three selected plants. The survey was performed bi-weekly from Jan 2003 to Dec 2004 with randomly selected plants, and from Jul 2003 to Dec 2004 for selected plants. We used the selected plants to determine whether or not there would be new chironomid larva infections after all the impounded water was extracted from these plants on each sample date. After 6 months of survey, three other selected plants were substituted because the leaves of the initially selected plants had become damaged either by sampling or natural senescense and no water could be impounded.

The water contained in the leaf axils of the plants was extracted with a 3 mL plastic pipette, placed separately in labelled plastic vials (200 mL) and transported to the laboratory where the number of chironomid larvae, pH and volume of water were determined. Weather data (temperature, rainfall and RH) were obtained from the Meteorological Station (Servicio Metereológico Nacional, Fuerza Aerea Argentina, Centro de Información Metereológica de Buenos Aires) in La Plata city. Samples were kept at 4 C until Harpellales fungi were identified and prevalence (percentage of infected chironomid larvae from randomly selected plants per sample date) was determined. Chironomids were preserved in 70 % ethanol and deposited with specialists for identification.

Larval dissection and culturing of Harpellales.--

Each chironomid larva was placed in a drop of sterile distilled water for dissection (according to Lichtwardt 1986) under a Zeiss Stemi DV4 stereomicroscope. Dissections were done by pulling away the abdomen and thorax from the head with fine forceps and separating the hindgut and midgut. The peritrophic matrix was separated from the midgut and its contents were cleared by holding either end of the membrane with forceps and lifting it few times from a water droplet on a glass slide. The cleared peritrophic matrix was mounted in a drop of sterile distilled water on a glass slide and

examined under phase contrast with an Olympus CH-30 microscope. The hindgut was opened with a fine needle and mounted separately from the peritrophic matrix. Semi-permanent mounts of peritrophic matrix and hindgut were prepared using lactophenol with 0.01 % cotton blue (w/v), the added cover glass was sealed to the slide with clear fingernail polish, and deposited in the fungal microscope slide collection at the CEPAVE herbarium.

Trichomycete thalli from infected larvae were used for attempts to isolate the fungus in BHIGTv medium (White et al 2006b). The medium consists of a 1:1 mixture of dilute brain-heart infusion (1/10 BHI) and tryptone glucose-salts (TG) media with vitamins (biotin and thiamine), covered with a shallow overlayer of sterile distilled water mixed with a penicillin-streptomycin antibiotic solution (individual formulae are provided in Lichtwardt et al 2001).

Data analysis.--

The non-parametric Kruskal-Wallis analysis was used to determine if there were significant differences in prevalence of Harpellales between seasons throughout the year on randomly selected plants. The Kruskal-Wallis analysis was also used to verify if there were significant differences in the number of chironomid larvae between seasons throughout the year on randomly selected plants. Seasons for the Southern Hemisphere are defined as follows: Spring, Sep 21--Dec 20; Summer, Dec 21--Mar 20; Fall, Mar 21--Jun 20 and Winter, Jun 21--Sep 20.

The relationship between prevalence, different environmental variables (temperature, rainfall, and RH), impounded water volume, pH, and chironomid larval density were analyzed by correlation.

RESULTS

Hosts.--

126 Two species of chironomid larvae were found living in the accumulated water from
127 *E. cabrerae* plants, *Polypedilum* sp. and *Metriocnemus erygiotelmatus* Donato & Paggi.
128 *Polypedilum* sp. (N = 2632) was present on all sample dates (N = 52) and found in 385
129 plants (74 %). *Metriocnemus erygiotelmatus* was less abundant (N = 162) and was
130 present on fewer sample dates (n = 25) than *Polypedilum* sp. The mean number of
131 chironomid larvae per sample date was not significantly different between seasons ($P >$
132 0.10) for both chironomid species.
133 *Trichomycete taxa.*--
134 Two species of Harpellales were found in the digestive tract of chironomid larvae
135 from *E. cabrerae* plants: *Stachylina lentica* White & Lichtwardt (Harpellales:
136 Harpellaceae) was attached to the peritrophic matrix that lines the midgut of both
137 species of chironomids, while *Smittium phytotelmatum* Lichtwardt (Harpellales:
138 Legeriomycetaceae) was only present in the hindgut of *Polypedilum* sp. Both species
139 represent the first report from Argentina, and *St. lentica* represents the first report from
140 phytotelmata. *Stachylina lentica* and *Sm. phytotelmatum* were present across a wide
141 range of measured independent variables (TABLE I).
142 *Prevalence.*--
143 *Randomly selected plants.*
144 Among the randomly selected plants, 3.82 % (n = 82) of all the *Polypedilum* larvae
145 were infected with *St. lentica* and 2.05 % (n = 54) were infected with *Sm.*
146 *phytotelmatum* throughout the two-year survey.
147 Over all the sample dates (N = 52), *St. lentica* was present 34.6 % (n = 18) of the
148 time and, of the total plants sampled (N = 520), this species was in 5.96 % (n = 31) of
149 them. The prevalence ranged between 0 to 30.2 %, with the highest values occurring in
150 the Summer of 2003 and in the Spring of 2004.

Smittium phytotelmatum was more abundant than *St. lentica,* being present in 6.35 % (n = 33) of the plants on 44.23 % (n = 23) of the sample dates. The prevalence varied between 0 to 25 %, with the highest values occurring during the Fall of 2003 and the late Summer of 2004 (FIG. 2).

The larvae of *M. eryngiotelmatus* only harbored *St. lentica* rarely (n = 3), and were not found with *Sm. phytotelmatum*. On one sample date, the percentage of infections with *St. lentica* was high (50 %) but only two *M. eryngiotelmatus* larvae were present. Due to the few occurrences of Harpellales in *M. eryngiotelmatus*, no statistical analysis was performed.

Selected plants.

Both *Sm. phytotelmatum* and *St. lentica* were present in selected plants as well (FIG. 3). The highest percentage of infection (for one plant) was 85.7 % for *St. lentica* and 50 % for *Sm. phytotelmatum*. In plant #1, *Sm. phytotelmatum* was present on four sample dates and varied between 0 to 50 % (for the higher, value the total number dissected, n, = 6) larval infection. *Stachylina lentica* was present on 7 sample dates and the infections ranged from 0 to 85.7 % (n = 7). In plant #2, *Sm. phytotelmatum* was present on 7 sample dates and the infection varied from 0 to 50 %, (n = 2), while *St. lentica* was present on 2 sample dates with a range of 0 to 33.3 % (n = 6) infection. In plant #3, *Sm. phytotelmatum* was present on 4 sample dates with a high value of 50 % (n = 16) infection, and *St. lentica* was present in 3 plants where the value of infection was 7.1 %. (n = 14).

Statistical analyses.--

Kruskal-Wallis analysis of the results showed that there were no significant difference in the prevalence of Harpellales from chironomid larvae between seasons (all $P > 0.10$, n = 7).

There were no correlations (all $P > 0.10$, n = 51) between prevalence and temperature (r = 0.02), impounded volume (r = 0.06), pH (r = 0.04), rainfall (r = 0.01) or larval density (r = 0.08). Neither were there significant correlations between impounded rainfall 2 wk before the sampling date and the mean volume of water per selected plant (r = 0.03). The volume had a low significant effect (r = 0.31) on larval density, whereas a very weak negative correlation (r = -0.12) was observed between temperature and the density of larvae per plant.

Cultures.--

Fungal thalli of *Sm. phytotelmatum* were successfully isolated from hindguts of *Polypedilum* sp. larva and axenic cultures were obtained and deposited in the Fungal Collection of CEPAVE (accession number: CEP-203). Similar attempts to isolate *St. lentica* were unsuccessful. To date *Stachylina* is a genus that has yet to be cultured.

DISCUSSION

Despite that our data show that the occurrence of *Sm. phytotelmatum* and *St. lentica* from *E. cabrerae* plants was not significantly different between seasons for both years, the infection was not constant over the year. A study of seasonality of trichomycetes in black flies from three streams in the USA by Beard and Adler (2002), is the only paper in which statistical differences for occurrence between season was demonstrated. They reported that the presence of *Harpella melusinae* (Harpellales: Harpellaceae) in *Simulium pictipes,* the host named later as *S. innoxium* (Adler et al 2004), varied by season but not by year in one of three sampled sites.

Smittium phytotelmatum and *St. lentica* had the highest occurrence during the Spring, Summer and Fall, except for the Spring of 2003 and Fall of the 2004 when the infection was low. *Stachylina lentica* infection was also relatively high (12.86 %) at the end of the Winter of 2003. These periods with high infections are similar to the results

obtained by Maciá et al (1995, 1997) in a study of parasites and pathogens from mosquitoes in Argentina and López Lastra et al (2003) who studied the temporal changes in the occurrence of Trichomycetes in dipteran hosts living in water in cemetery flower vases. In the studies by Taylor et al (1996) and Beard and Adler (2002) on Trichomycetes in black flies from streams, the authors reported a high prevalence of *Harpella melusinae* (Harpellales: Harpellaceae) during the Northern Hemisphere Summer and Fall.

Generally, when a plant contained infected larvae, most chironomid larvae were infected in such plant. This may have been due to the impounded water in each plant being continuous, resulting in spreading of the inoculum and infection of larvae present in the leaf axils. However, due to the overall low number of randomly selected plants with infected chironomids present for each sample date, the prevalences of Trichomycetes in our study were very low. In previous reports from other environments, El Sheriff (1975) and Beard and Adler (2002) recorded 100 % infection with *H. melusinae* in black flies (Diptera: Simuliidae) over the entire sampling year, while Taylor et al (1996) reported *H. melusinae* in simuliids ranging from 80 to 100 % during most of the year.

Phytotelmata are typically unstable habitats, with desiccation occurring for short periods of time and requiring the plants to refill with water in order to be colonized by midges, and presumably by trichomycete fungi. The survey of selected plants allowed us to verify sporadic infections of chironomid larvae during the year, even after periods of dessication. Goettel (1987) and Grigg and Williams (1989) also observed *Smittium* species at sites that had been dry for extended periods.

Neither the environmental variables (temperature, rainfall and RH) or the densities of larvae nor the volume of impounded water volume or pH affected the prevalence of

Harpellales. We did not find any new infections between Mar and Oct 2004, so we hypothesized that transmission occurs more readily during warmer months; however, inoculum may persist in the impounded water or as inhabitants of chironomid larvae during colder periods. The life cycle of *Polypedilum* sp. in *E. cabrerae* plants is poorly known, but we think that, as occurs in other insects (Bradshaw and Holzapfel 1977, Copeland and Craig 1989), chironomids may survive as larvae during the colder periods in the plants. Also, trichospores of *Sm. phytotelmatum* may survive for a few months in cold water as previously reported for *Sm. culisetae* (Williams 1983). No sexual activity was observed in either species of Harpellales during our study, and consequently no zygospores were detected. The infection of chironomids that was observed in selected plants during the Winter of 2003 may have occurred due to remaining inoculum from the previous season.

The Harpellales are known to be widespread worldwide but the means by which they disperse among sites has not been as well studied, particularly in ephemeral habitats. During our study the river flooded and inundated the plants on more than one occasion, and could have been a source of fungal inoculum and chironomid larvae, because we have found some *Polypedilum* sp. larvae infected with *Sm. phytotelmatum* from temporary ponds near our sample site. Lichtwardt (1994) and Reeves (2004) also have suggested that reinfestation of fungi by insects in this kind of environment may be aided by phoretic dispersal by other animals that visit phytotelm plants.

We did not examine midge adults or released eggs. However, the eggs of gravid black flies can be partly or totally replaced by fungal chlamydospores (=ovarian cysts), which are then deposited into the larval environment by the adult female during oviposition (Nelder et al 2003, Rizzo & Pang 2005). Moss (1998) provided evidence of chlamydospores of *Smittium* species attached to mucilage covering chironomid eggs.

Trichospores that develop on such released chlamydosporic stages serve as new inoculum when newly emerged larvae ingest them (Lichtwardt 1996, White et al 2006a).

Clearly, there are questions that remain to be answered about the biology and ecology of these endobionts. As the first study to indicate the presence of Harpellales infecting insects in phytotelmata over time, it is hoped that the evident adaptability of these symbionts in such unique lentic habitats will stimulate further studies of larval insects in similar repositories.

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FIG. 1: *Eryngium cabrerae* plant.

FIG. 2. Prevalence of Harpellales in *Polypedilum* sp. larvae from randomly selected *E. cabrerae* plants.

FIG. 3: Occurrence of Harpellales in repeatedly sampled selected plants. Arrows denote the date that originally selected plants were each replaced with a substitute plant.

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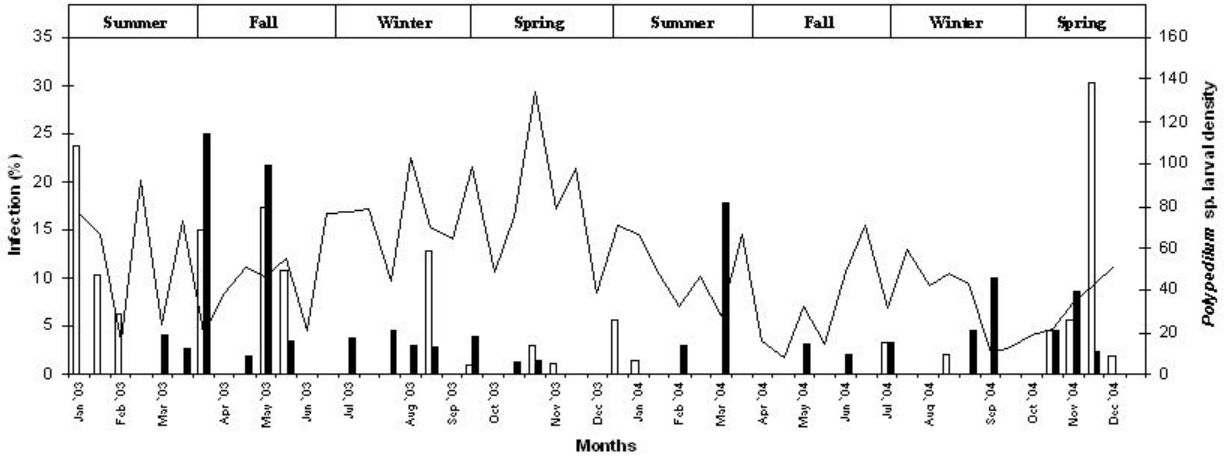
Stachylina lentica
Smittium phytotelmatum
Polypedilum sp.
Summer
Fall
Winter
Spring
Summer
Fall
Winter
Spring
Infection (%)
Polypedilum sp. larval density
Months
Jan '03
Feb '03
Mar '03
Apr '03
May '03
Jun '03
Jul '03
Aug '03
Sep '03
Oct '03
Nov '03
Dec '03
Jan '04
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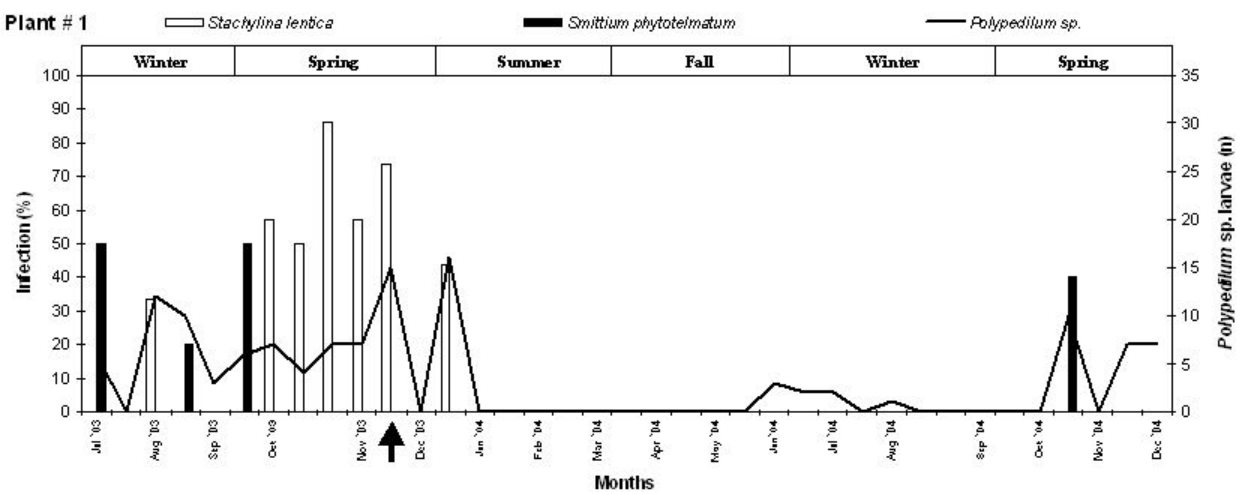
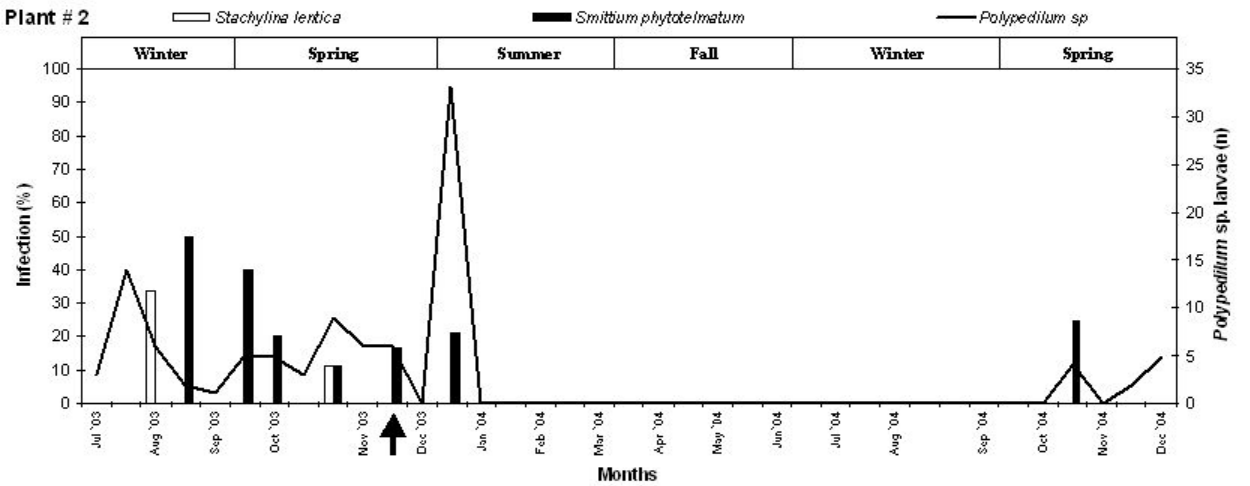

Plant # 1
Stachylina lentica
Smittium phytotelmatum
Polypedilum sp.
Winter
Spring
Summer
Fall
Winter
Spring
Infection (%)
Polypedilum sp. larvae (n)
Jul '03
Aug '03
Sep '03
Oct '03
Nov '03
Dec '03
Jan '04
Feb '04
Mar '04
Apr '04
May '04
Jun '04
Jul '04
Aug '04
Sep '04
Oct '04
Nov '04
Dec '04
Months


Plant # 2
Stachylina lentica
Smittium phytotelmatum
Polypedilum sp
Winter
Spring
Summer
Fall
Winter
Spring
Infection (%)
Polypedilum sp. larvae (n)
Jul '03
Aug '03
Sep '03
Oct '03
Nov '03
Dec '03
Jan '04
Feb '04
Mar '04
Apr '04
May '04
Jun '04
Jul '04
Aug '04
Sep '04
Oct '04
Nov '04
Dec '04
Months

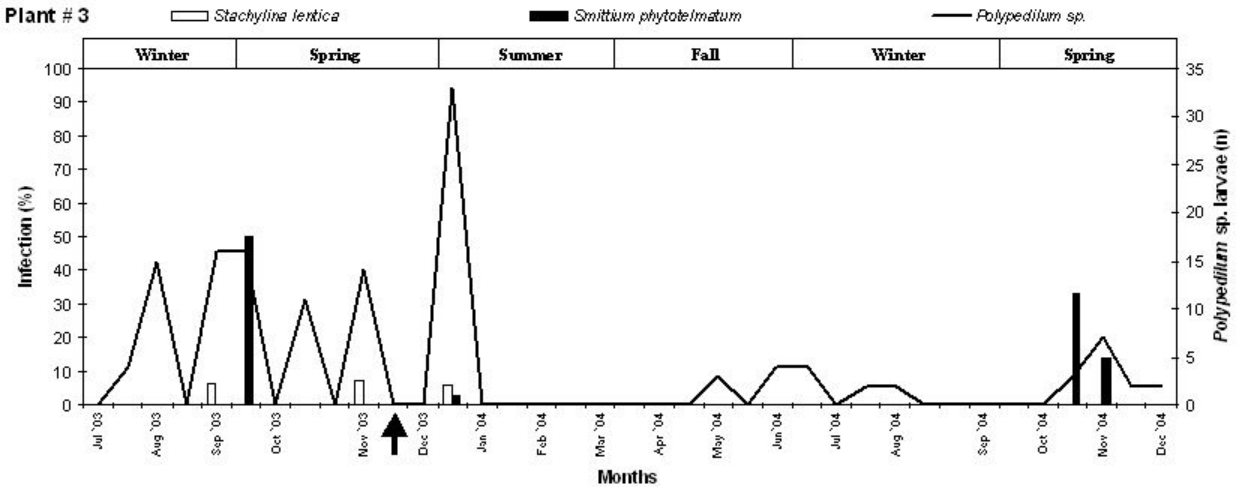

Plant # 3
Stachylina lentica
Smittium phytotelmatum
Polypedilum sp.
Winter
Spring
Summer
Fall
Winter
Spring
Infection (%)
Polypedilum sp. larvae (n)
Jul '03
Aug '03
Sep '03
Oct '03
Nov '03
Dec '03
Jan '04
Feb '04
Mar '04
Apr '04
May '04
Jun '04
Jul '04
Aug '04
Sep '04
Oct '04
Nov '04
Dec '04
Months

TABLE I. Independent variables within which Harpellales were present in *E. cabrerae* plants.

	Range of mean temp (ºC)[1]	Range of mean RH (%)[1]	Range of mean rainfall (mL)[1]	Ranged of volume (mL)[2]	Range of density larvae (n)[2]	Range of pH[2]
Sm. phytotelmatum	3,9 to 30,1	52,4 to 95,7	0 to 65	5 to 60	1 to 28	7,07 to 8,84
St. lentica	5,1 to 29,7	52,4 to 95 ,7	0 to 64	5 to 70	1 to 26	7,07 to 8,40

[1] Accumulated for 2 week periods before actual collection

[2] For actual dates of sampling.