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Assessing winter survival of *Pandora neoaphidis* in soil applying bioassay and molecular approaches

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Abstract: *Pandora neoaphidis* (Entomophthorales) is one of the most important fungal pathogens of aphids and it has a great potential for use in biocontrol. As cultivation of *P. neoaphidis* is difficult, conservation biocontrol strategies are favoured. However, little is known on overwintering strategies of this fungus. It is hypothesized that natural areas may play an important role for survival and that undisturbed soil may serve as inoculum source for new populations in spring. To test these hypotheses, we have developed a cultivation-independent PCR-based diagnostic tool that allowed for detection of *P. neoaphidis* DNA in top soil samples collected during winter from a nettle field harboring infected aphids in fall. Results suggested an overwintering stage of *P. neoaphidis* in top soil layers. The PCR-based method, however, does not provide information on viability or virulence of detected *P. neoaphidis* material. Therefore, a field study was initiated in summer 2006 which will last until spring 2007. It aims at investigating winter survival of *P. neoaphidis* in top soil layers and to test whether *P. neoaphidis* material detected with the molecular tool represents infectious fungal material. For this purpose, molecular analyses of soil samples are accompanied with a bioassay in which aphids are placed on soil samples and *P. neoaphidis* infection is monitored, and recorded as aphid mortality. The experimental layout consists of eight replicated caged plots (0.16 m^2) for each of four different treatments: 1) *paf* plots: lucerne plants inoculated with pea aphids and the fungus *P. neoaphidis*; 2) *pa* plots: lucerne plants with aphids but without artificial *P. neoaphidis* inoculation; 3) *p* plots: lucerne plants without aphids and *P. neoaphidis*; and 4) *bs* plots: bare soil, which was covered with a weed barrier fabric. Soil samples were collected at four different time points in 2006. To date, bioassays and molecular analyses were carried out on soil samples from *paf* plots. Our preliminary results indicate a good correlation between bioassay data and PCR-based data, and suggest a decrease of *P. neoaphidis* inoculum in soil after winter begins.

Key words: *Pandora neoaphidis*, molecular detection, conservation biocontrol, winter survival, bioassay

Introduction

Pandora neoaphidis (Remaudière and Hennebert; Zygomycota, Entomophthorales) is one of the most important fungal pathogens infecting aphids (Homoptera: Aphidoidea) in temperate areas (Keller and Suter, 1980). This aphid-specific fungus has been reported to cause natural epizootics, which can dramatically reduce host populations (e.g. Keller and Suter, 1980; Feng et al., 1991). However, natural epizootics often occur too late to reduce aphid populations below the damage threshold (Keller, 1998; Keller and Suter, 1980).

P. neoaphidis has a great potential for use in biological control of aphids. Two approaches i.e. inundation and inoculation biocontrol have been investigated in various studies, but they have shown limited effectiveness (Wilding, 1981; Wilding et al. 1990; Shah et al., 2000). Conservation biocontrol, which is defined as a "*modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests*" (Eilenberg et al., 2001), is a promising third approach for the control of aphids with *P. neoaphidis*. To implement such conservation biological control strategies, detailed knowledge on the life cycle and ecology of the pathogen are prerequisites. However, many of

these aspects are only poorly understood for *P. neoaphidis*. Especially, knowledge on overwintering sites and mechanisms, as well as the initiation of infection in spring is very limited. It is hypothesized that natural areas may play an important role for the winter survival of *P. neoaphidis* (Keller, 1998) and that undisturbed soil may serve as inoculum source for new populations in spring (Nielsen et al., 2003).

We developed a cultivation-independent PCR-based diagnostic tool that allows for a specific, sensitive, and fast detection of *P. neoaphidis* DNA in various environmental samples including infected aphid cadavers, soil samples, living plant material, and plant debris (Fournier et al., in preparation). This tool consists of species-specific primer pairs that target sequences in the rRNA gene cluster of *P. neoaphidis*. The application of this tool to soil samples collected during winter 2004/2005 from a nettle field harbouring infected aphids in 2004, suggested an overwintering stage of *P. neoaphidis* in top soil layers. Although such a PCR-based diagnostic tool may offer great advantages because of its cultivation-independence and sensitivity, it does not provide information on the viability or the virulence of the fungal material detected in the environment.

The aims of this follow-up study were to investigate the winter survival of *P. neoaphidis* in top soil layers and to test whether *P. neoaphidis* material detected with the molecular tool represents infectious fungal material. For this purpose, a field experiment was initiated in August 2006 with caged naturally and artificially infected aphid populations. *P. neoaphidis* infection was monitored and the presence of the fungal inoculum in top soil layers was investigated with a bioassay as well as with the cultivation-independent PCR-based approach.

Material and methods

Experimental layout

The plot experiment was established, in a grass-clover field at Agroscope Reckenholz-Tänikon Research Station ART (Zurich, Switzerland) in summer 2006. The experimental layout consists of 32 plots of 0.16 m^2 arranged in a complete randomized block design with 8 replicates of 4 treatments. The spacing between two plots was of 1.5 m. The four types of treatments were designed as follows: 1) *paf* plots: 16 lucerne plants inoculated with approximately 1500 healthy pea aphids (*Acyrthosiphon pisum*) (released on October 2) and 150 aphids that were infected with the fungus *P. neoaphidis* (released on October 10 and 16); 2) *pa* plots: Lucerne plants with aphids but without artificial *P. neoaphidis* inoculation; 3) *p* plots: Lucerne plants without aphids and *P. neoaphidis*; and 4) *bs* plots: bare soil. *p*, *pa*, *paf* plots were caged with a 200 µm Nitext® mesh fabric (Sefar, Heiden, Switzerland) to avoid insect transit, whereas soil of *bs* plots was covered with a weed barrier fabric ('GrowStop', Windhager, Thalgau, Austria)

Monitoring of aphid populations and prevalence of infection

After releasing the aphids infected with *P. neoaphidis* into *paf* plots, the aphid population as well as the percentage of *P. neoaphidis* infected aphids was monitored at three time points (October 22, October 29, December 10) in *paf* and *pa* plots. The aphid population was estimated by counting all aphids present on one plant per plot. To estimate the prevalence of *P. neoaphidis* infection per plot, 50 3rd to 4th instar aphid nymphs that did not display infection symptoms were collected from each plot and were transferred to individual faba bean (*Vicia faba*) plants in pots wrapped with cellophane bags (Celloclair AG, Liestal, Switzerland). After 5 days of incubation (18°C with a 16:8 L:D), the number of aphids that died from *P. neoaphidis* infection was determined.

Monitoring of P. neoaphidis in the soil

Top soil samples (top 1 cm soil layer) were collected from every plot at four different time points in 2006: 1) on October 2, just before releasing aphids into *pa* and *paf* plots; 2) on November 2, after high levels of infection with *P. neoaphidis* were observed in *paf* plots and in *pa* plots; 3) on November 21, after the first nights with temperatures below freezing; and 4) on December 13, when no more living aphids were observed in the *paf* and *pa* plots.

Bioassay: The soil samples were transferred to 10 cm Petri dishes without disturbing the soil structure. After 24 h incubation at 18°C with a 16:8 L:D photoperiod, the soil samples were screened for the presence of *P. neoaphidis* by performing a bioassay: Approximately 100 *A. pisum* aphids of all developmental stages originating from a laboratory culture were introduced to each soil sample and incubated for 14h at 18°C in the dark. Subsequently, 20 3rd to 4th instar nymphs per Petri dish were transferred to a bean plant. After 7 days of incubation, the number of aphids that died from *P. neoaphidis* infection (*P. neoaphidis* cadavers) was determined. Mortality was calculated according to Feng et al., 1991 as: mortality (%) = [(number of *P. neoaphidis* cadavers)/(live aphids + *P. neoaphidis* cadavers)] x 100. For each time point, averages were calculated for the 8 replicates per treatment.

PCR-based detection: 500 mg of soil were collected from each petri dish immediately after collection of the top soil and metagenomic DNA was extracted according to Bürgmann et al. (2001). The PCR-based detection of *P. neoaphidis* was performed using a pair of specific primers targeting sequences in the rRNA gene cluster of *P. neoaphidis* (Fournier et al., in preparation).

Results and discussion

Monitoring of aphid populations and prevalence of infection

On October 22, less than two weeks after the inoculation of *paf* plots with *P. neoaphidis* infected aphids, the average aphid population in *paf* plots was estimated at 6'700, and the prevalence of *P. neoaphidis* infection reached an average of 88% per plot. At the same date, the average aphid population in *pa* plots was 8'200 and the prevalence of infection with *P. neoaphidis* was 16%. Even tough *pa* plots were not artificially inoculated with the fungus, it rapidly established itself in these plots. The origin of the fungal material that infected the aphids in the *pa* plots is not known. This material may have originated from the *paf* plots or from the surrounding fields.

On October 29, due to high prevalence of *P. neoaphidis* infection, the aphid population in *paf* plots has dropped to less than 900 individuals per plot, and the prevalence of infected aphids was 90%. The average aphid population in *pa* plots increased to 11'000 individuals and the prevalence of infection reached 68%. On December 10, no more living aphids were present in *pa* and *paf* plots.

Bioassay and PCR-based detection

Currently, bioassays and PCR-based analyses were performed with all soil samples collected from *paf* plots in 2006 (Figure 1). *P. neoaphidis* was neither detected with the bioassay nor with the PCR-based method in the soil samples collected from the *paf* plots on October 2 (before releasing the aphids in *pa* and *paf* plots). In the *paf* soil samples collected on November 2 (approximately 2 weeks after releasing *P. neoaphidis* in *paf* plots) an average aphid mortality of 47% was recorded with the bioassay, and signals (6 strong, 2 weak) of PCR products of the expected size were detected in all eight replicates. On November 21, the bioassay aphid mortality was 44% and PCR signals (7 strong, 1 weak) were detected in all *paf* samples. On December 13, when no more living aphids were present in the plots, the bioassay

aphid mortality in the bioassay dropped to 1% and overall PCR signals were weaker than on November 2 and 21. Positive signals were detected in 6 samples of the 8 replicates (2 strong, 4 weak).

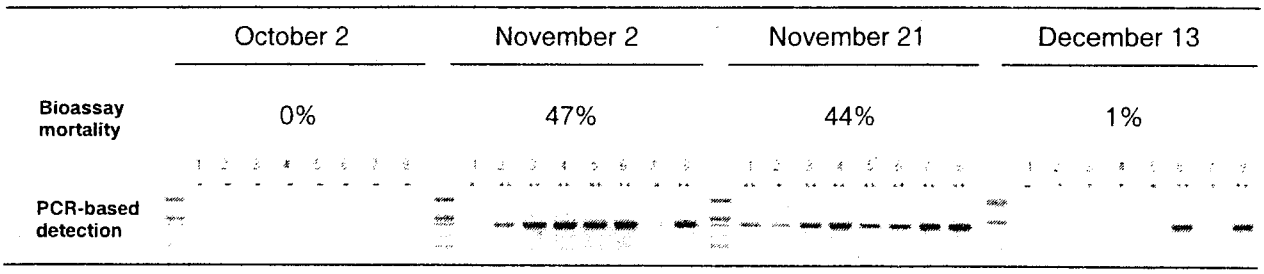

Figure 1. Results of bioassays and PCR-based analyses performed with soil samples collected from *paf* plots between October and December 2006. A) Bioassay results, expressed as average percent mortality of the eight replicate soil samples collected from *paf* plots at each time point. B) PCR-based detection, obtained with specific amplification of a targeted sequence from the rRNA gene cluster of *P. neoaphidis*. '++': strong signal of PCR product; '+': weak signal; '–': no signal.

Monitoring of the aphid population and the prevalence of *P. neoaphidis* infection allowed to determine that several thousands of aphids were infected with *P. neoaphidis* in every *pa* and *paf* plot at the end of October 2006. At this time point the prevalence of *P. neoaphidis* in *paf* plots was so high (90%) that the aphid population was strongly reduced (<1000 aphids/plot). This demonstrated the potential of *P. neoaphidis* to control aphid populations. The fungal material that was generated could be detected in the soil of all *paf* plots with the bioassay as well as with the PCR-based method. Our results show a correlation between the bioassay data and the PCR-based data obtained from the analyses the *paf* soil samples collected at all four different time points, suggesting that *P. neoaphidis* material detected by PCR may represent infectious material. Data revealed that a high level of *P. neoaphidis* inoculum was present in the soil of *paf* plots in November 2 and 21 (after a high *P. neoaphidis* prevalence was detected in these plots) but that the level of inoculum strongly decreased until December 13 (at the beginning of winter and at a time when no more living aphids were present in the plots). These preliminary results suggest a strong decrease of *P. neoaphidis* inoculum in the soil during winter. The results of additional bioassay experiments (in spring 2007), as well as the molecular analysis of all soil samples, will provide further information about the role of soil as a matrix for winter survival of *P. neoaphidis*.

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