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Assessing winter-survival of *Pandora neoaphidis* in soil with bioassays and molecular approaches

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ABSTRACT

Pandora neoaphidis (Entomophthoromycotina, Entomophthorales) is a specific pathogen of aphids with a great potential for use in biological control. The development of effective biological control strategies requires detailed knowledge of its biology and ecology. However, little is known on the overwintering strategies of this fungus. It is believed that natural areas may play an important role for survival and soil may serve as an inoculum source for new populations in spring. This study aimed to investigate winter-survival of *P. neoaphidis* in topsoil layers in a field experiment by assessing fungal persistence and potential to infect and control pea aphid (*Acyrthosiphon pisum*) populations in spring. For this purpose, a selected *P. neoaphidis* strain was introduced in the form of living infected pea aphids into caged plots containing defined pea aphid populations. Within 3 weeks the aphid populations decreased massively and a significant amount of *P. neoaphidis* inoculum accumulated on the soil providing optimal conditions for investigating the winter-survival of this fungus. Prevalence and pathogenicity of *P. neoaphidis* in plot soils were assessed from fall 2006 until spring 2007 using a combination of bioassay, quantitative PCR, and single nucleotide polymorphism genotyping approaches. Results indicated that the introduced strain did not survive the winter on the soil and that the strains present in the plots in the following spring were derived from outside the plots. However, the introduced strain had a beneficial effect on plant survival. The tools developed and applied in this study proved powerful and reliable for tracking specific target strains of *P. neoaphidis* in the environment.

1. Introduction

Aphids can cause severe crop losses by inducing feeding injuries on plants (Quisenberry and Ni, 2007) and by transmitting plant viruses (Katis et al., 2007). *Pandora neoaphidis* (Remaudière & Hennebert) Humber (Entomophthoromycotina, Entomophthorales) is an important fungal pathogen of aphids in temperate regions and it has great potential for use in biological control. It is aphid-specific (Keller, 1991), infects more than 70 aphid species (Pell et al., 2001), and can cause epizootics that dramatically reduce aphid populations (Feng et al., 1991; Keller and Suter, 1980; Steenberg and Eilenberg, 1995). *In vitro* cultivation of this fungus is difficult and large scale production of biological control strains has not been successful so far (Shah and Pell, 2003). During the last decades, interest in the use of *P. neoaphidis* in conservation biological control strategies has grown (Barta and Cagan, 2003; Baverstock et al., 2008; Ekesi et al., 2005; Fournier et al., 2008; Keller, 1998; Pell et al., 2001; Powell and Pell, 2007). The general aim of this strategy is to modify habitats to increase the occurrence, and therefore the efficacy of biocontrol organisms to control pests (Eilenberg et al., 2001). It has been suggested that management practices such as irrigation (increased moisture), reduced pesticide applications, and establishment of overwintering sites for possible alternative hosts may enhance abundance of entomophthoralean fungi for biological control of aphids (Pell et al., 2001). However, successful use of *P. neoaphidis* in such strategies requires a thorough knowledge of its biology and the environmental factors influencing its presence and survival (Nielsen et al., 2003).

Many aspects of the life cycle of *P. neoaphidis* are only poorly understood. In particular, knowledge of overwintering stages and sites as well as the factors that trigger initiation of infection in spring is very limited (Nielsen et al., 2008). It has been suggested that soil may play an important role in winter-survival of *P. neoaphidis* and may serve as the inoculum source for new aphid populations in spring (Baverstock et al., 2008; Fournier et al., 2008; Keller, 1998; Nielsen et al., 2003). Furthermore, it has been proposed that natural and semi-natural landscape elements such as field margins, nettle patches, and natural meadows may provide overwintering sites and reservoirs for aphid-pathogenic entomophthoralean fungi (Barta and Cagan, 2003; Baverstock et al., 2008; Ekesi et al., 2005; Keller and Suter, 1980; Shah et al., 2004).

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However, detailed knowledge of the overwintering ecology of *P. neoaphidis* is still missing.

The development of biological control agents (BCAs) including application and quality control strategies requires tools to detect, genotype, and quantify the BCAs. Two approaches have been used for detecting *P. neoaphidis* in soil. One approach is based on baiting the fungus using bioassays (Baverstock et al., 2008; Latteur, 1980; Nielsen et al., 2003) and the other approach relies on detection with cultivation-independent conventional end-point PCR (Fournier et al., 2008; Tymon et al., 2004). Both approaches have specific advantages and disadvantages, which make them complementary. On the one hand, bioassays provide information on viability and pathogenicity of the detected fungi. On the other hand, they are labor intensive and may lack robustness as the factors involved in the infection process are not yet fully understood (Baverstock et al., 2008; Nielsen et al., 2003, 2008). The PCR-based approach is faster and more robust as it is based on well-established protocols for DNA extraction and PCR amplification. However, DNA-based cultivation-independent tools do not provide information on viability and pathogenicity of the detected organisms (Enkerli and Widmer, 2010; Nielsen et al., 2008). Both detection methods have been applied to investigate overwintering of *P. neoaphidis* and have suggested survival of the fungus in topsoil layers (Baverstock et al., 2008; Fournier et al., 2008; Nielsen et al., 2008).

Genotyping tools allow investigation of population structures in the environment and therefore may help to provide basic information on the biology and ecology of a potential BCA (Anderson and Cairney, 2004). Moreover, such tools may be crucial for characterization of specific BCA strains when assessing specificity and virulence during strain selection processes (Bidochka, 2001) or when investigating persistence of specific strains in the environment (Enkerli et al., 2004; Schwarzenbach et al., 2007). Various genetic tools have been applied to characterize *P. neoaphidis* strains, including randomly amplified polymorphic DNA (Williams et al., 1990), enterobacterial repetitive intergenic consensus PCR (Versalovic et al., 1991), and inter-simple-sequence repeat PCR (Zietkiewicz et al., 1994). Although these methods have allowed detection of intraspecific variation among *P. neoaphidis* isolates (Nielsen et al., 2001; Rohel et al., 1997; Tymon and Pell, 2005; Tymon et al., 2004), they cannot be applied directly to complex DNA extracts obtained from environmental samples due to the lack of target specificity of the primers. Therefore, laborious and time-consuming isolation and cultivation of the organisms of interest are required. Approaches based on ribosomal internal transcribed spacer (ITS) size, restriction, and sequence analyses have been applied in various studies and can be used without prior cultivation steps (Fournier et al., 2008; Nielsen et al., 2001; Rohel et al., 1997; Tymon et al., 2004). However, these approaches have not allowed to consistently discriminate *P. neoaphidis* at the intraspecific level. Recently, a molecular assay has been developed that targets single nucleotide polymorphisms (SNPs) distributed among different genes or genomic regions of *P. neoaphidis* (Fournier et al., in press). SNPs are commonly detected by single-base extension of oligonucleotide primers adjacent to the SNP sites, and they typically display two alleles (Brookes, 1999). This genotyping tool has been reported as powerful for typing *P. neoaphidis* isolates (Fournier et al., in press). Application of the SNP assay has allowed discrimination of *P. neoaphidis* strains with high resolution. It is applicable to DNA extracts obtained from *P. neoaphidis* cultures, as well as from fungal-killed aphid cadavers, and therefore allows for cultivation-independent genotyping of *P. neoaphidis* in the environment. Fournier et al. (in press) have observed that at a given locus the two alleles can be present simultaneously in *P. neoaphidis*, which they referred to as mixed alleles.

Quantification of fungal species in the environment has traditionally relied on the use of selective media (Lievens et al., 2005). This approach does not allow for quantification of organisms that cannot be cultivated or are difficult to cultivate as in the case for *P. neoaphidis* (Papierok and Hajek, 1997). Quantification techniques that are based on quantitative PCR (Heid et al., 1996) allow to circumvent this problem as no cultivation step of the fungus is required prior to quantification (Bustin, 2004). Approaches based on quantitative PCR have successfully been implemented to quantify entomopathogenic fungal species such as *Beauveria brongniartii* (Saccardo) Petch (Schwarzenbach et al., 2009) and *Entomophaga maimaiga* Humber, Shimazu, & Soper (Castrillo et al., 2007), or specific strains of *Beauveria bassiana* (Balsamo) Vuillemin (Bell et al., 2009; Castrillo et al., 2008) and *Metarhizium anisopliae* var. *acridum* Driver and Milner (Bell et al., 2009) in host insects or soil. Such approaches for quantification of *P. neoaphidis* in the environment have not been established yet.

The goal of our study was to determine the ability of *P. neoaphidis* for winter-survival in topsoil layers and to assess its potential to infect and control aphid populations in a field experiment. A *P. neoaphidis* strain was applied in caged field plots containing healthy aphids in fall 2006. Abundance and infection rate of the inoculum were monitored until spring 2007 using a bioassay, SNP-based genotyping, and a quantitative PCR approach developed in this study.

2. Materials and methods

2.1. Plant culture

Lucerne plants (*Medicago sativa* Linnaeus, cv. Sanditi) used in the field experiment were grown from seeds germinated between layers of water-saturated filter paper in 16 h light and 8 h dark at 20 °C. Seedlings were planted in pots (9 cm diameter, 7 cm deep) containing commercial turf soil (Ricoter Erdaufbereitung AG, Aarberg, Switzerland) and grown in the greenhouse for 3 weeks in 16 h light at 17 °C and 8 h dark at 13 °C. Subsequently, the lucerne plants were acclimatized to outdoor conditions for 2 weeks in the shade before transplanting them into the field plots.

Broad bean plants (*Vicia faba* Linnaeus, cv. Sirocco) were grown from seeds in an incubation chamber in 16 h light and 8 h dark, at 18 °C with 65–75% humidity in $35 \times 22 \times 5$ cm seed trays (50 seeds/tray) filled with autoclaved (121 °C, 25 min) multi-purpose compost (Obi-Ter, Märwil, Switzerland). Two- to three-week-old plants were transplanted into either 'large pots' (13 cm diameter, 11 cm deep) or 'small pots' (8 cm diameter, 8 cm deep). The 'large pots' were used for propagation of aphids for field release. They were filled with autoclaved multi-purpose compost and planted with six *V. faba* plants. The 'small pots' were used for monitoring aphid infection and production of infected aphid cadavers. They were prepared by pouring a 5-cm layer of 1% agar into the pot and transplanting one individual *V. faba* plant into a hole (1 cm diameter, 5 cm deep) that was made in the center of the solidified agar piece of each pot. Twenty milliliters of water were poured into the hole and a disc of parafilm (8 cm diameter, with a 5-mm hole in the center for the plant) was placed on the top of the agar before adding a 0.5-cm layer of sieved (2 mm) and gamma-sterilized soil (40 kGy, Studer Hard, Däniken, Switzerland). This setup allowed plant maintenance for 1 week without watering and therefore to circumvent disturbance and contamination of the aphids placed on the plants.

2.2. Aphid culture

Four clonal cultures of pea aphids (*Acyrthosiphon pisum* Harris, Homoptera: Aphididae) were used. One culture had been maintained for more than 20 years in the laboratory (culture 1) and