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Diversity of entomopathogenic Hypocreales in soil and phylloplanes of five Mediterranean cropping systems

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ABSTRACT

The diversity of entomopathogenic Hypocreales from the soil and phylloplanes in five Mediterranean cropping systems with different degrees of management [organic olive orchard conventional olive orchard, holm oak reforestation, holm oak dehesa (a multifunctional agro-sylvo-pastoral system), and sunflower plantation] was studied during four seasons. A total of 697 entomopathogenic fungal isolates were obtained from 272 soil samples, 1608 crop phylloplane samples and 1368 weed phylloplane samples. The following nine species were identified: *Beauveria amorpha, B. bassiana, B. pseudobassiana, B. varroae, Metarhizium brunneum, M. guizhoense, M. robertsii, Paecilomyces marquandii* and *lilacinum* using EF-1α gene sequences. All the fungal entomopathogenic species were found in both the soil and phylloplane samples, with the exception of *M. robertsii*, which was only isolated from the soil. The species richness, diversity (Shannon–Wiener index) and evenness (Pielou index) were calculated for each cropping system, yielding the following species ranking, which was correlated with the crop management intensity: holm oak reforestation > organic olive orchard > conventional olive orchard > holm oak dehesa > sunflower plantation. The number of fungal species isolated was similar in both phylloplane habitats and dissimilar between the soil and the crop phylloplane habitats. The ISSR analysis revealed high genotypic diversity among the *B. bassiana* isolates on the neighbourhood scale, and the isolates were clustered according to the habitat. These results suggest that the entomopathogenic Hypocreales in the phylloplane could result from the dispersal of fungal propagules from the soil, which might be their habitat of origin; a few isolates, including EABb 09/28-Fil of *Beauveria bassiana*, inhabit only the phylloplane.

1. Introduction

Entomopathogenic Hypocreales are distributed worldwide in almost all terrestrial ecosystems and habitats, in which they cause natural infections in many insect and mite hosts (Aira et al., 2007; Inglis et al., 2001). Until recent years, the contribution of their biodiversity on the regulation of pest populations was not investigated. Recently, considerably more ecological studies on entomopathogenic Hypocreales have been conducted to understand how agricultural practices affect their pathogenicity (Meyling and Eilenberg, 2007).

Entomopathogenic Hypocreales have typically been isolated from soil, which is considered their natural reservoir (Jaronski, 2010; Klingen and Haukeland, 2006). Differences in the natural occurrence, prevalence, and distribution of these fungi in soil have been reported to be related to the crop type and management (Bidochka et al., 1998; Meyling and Eilenberg, 2006b; Quesada-Moraga et al., 2007). Some specific Hypocreales species are able to interact with plant roots as rhizosphere-competent microorganisms, suggesting that they could survive in the soil without arthropod hosts (Hu and St. Leger, 2002; Klingen et al., 2015). Plant-fungi interactions have been reported for entomopathogenic Hypocreales, with the fungi acting as endophytes or epiphytes in the phylloplanes of several crop and weed species (Inglis et al., 1993; Meyling and Eilenberg, 2006a; Posada and Vega, 2005; Quesada-Moraga et al., 2006). Natural occurrences of entomopathogenic Hypocreales have been found in various plant parts, including the leaves, stem and roots of *Coffea arabica*, *Zea mays*, and *Oryza sativa* (Vega et al., 2008; Arnold and Lewis, 2005; Tian et al., 2004). Infection could result from phylloplane deposits through the endophytic pathway. By spraying plant leaves or dipping plant roots, tissue culture rhizomes or seeds in a fungal suspension or by

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a fungal soil drench, several authors have recently confirmed that entomopathogenic Hypocreales could enter plants. Seedling and stem injections of a fungal suspension are possible (Posada and Vega, 2005; Posada et al., 2007; Akello et al., 2007; Parsa et al., 2013; Keyser et al., 2014). Vertical transmission of entomopathogenic Hypocreales in the interior of plants following treatment of the seeds has recently been demonstrated (Quesada-Moraga et al., 2014). The fungus is able to colonize the seeds and grow inside the plants and, possibly, in the new seeds, conferring systemic protection to the plant.

Studies on the diversity of fungi, principally endophytes and entomopathogens, frequently use ecological indices to determine the importance of the entire community (Aung et al., 2008; Espinosa-Garcia and Langenheim, 1990; Gamboa and Bayman, 2001). These evaluations are frequently based on morphological characteristics and are insufficient to accurately determine the biodiversity within ecological communities (Meyling and Eilenberg, 2007). Molecular tools facilitate the accurate classification of entomopathogenic fungal species, and they could be used to identify the differences between closely related species that co-occur in an ecosystem (Hibbett et al., 2007; Rehner et al., 2011; Schneider et al., 2011; Webb et al., 2002).

Studies on the genetic diversity of entomopathogenic Hypocreales have typically been performed using isolates from culture collections (Garrido-Jurado et al., 2011), by infecting a certain pest (Aquino de Muro et al., 2005; Rehner et al., 2006), by focusing on one habitat (Hollingsworth et al., 2011; Schneider et al., 2012; Inglis et al., 2008; Meyling and Eilenberg, 2006a,b), or by collecting specimens from regions worldwide (Freed et al., 2011). None of these studies has simultaneously increased the understanding of species ecology on a local scale and in habitats in different ecosystems (Meyling and Eilenberg, 2007).

In this study, we aimed to investigate the diversity of enomopathogenic Hypocreales from the soil and phylloplanes in five Mediterranean cropping systems that have different degrees of management [an organic olive orchard, a conventional olive orchard, a holm oak reforestation, a holm oak dehesa (a multifunctional agro-sylvo-pastoral system), and a sunflower plantation] during four seasons. The isolates were identified using partial sequencing information from the EF-1α region, and the genetic diversity was determined by using inter-simple sequence repeat (ISSRs) to study the correlations between the cropping system, habitat, and time of isolation.

2. Materials and methods

2.1. Field sites and fungal isolation

The soil and the phylloplanes of the crop and of the weeds in the crop were sampled in five Mediterranean cropping systems with different degrees of management [an organic olive orchard, a conventional olive orchard, a holm oak reforestation, a holm oak dehesa (a multifunctional agro-sylvo-pastoral system), and a sunflower plantation] in three provinces of Andalucia (Supplementary Table S1). The conventional olive orchard (38°02′03.00″N, 4°30′29.00″W) and sunflower plantation (37°51′57.00″N,4°44′47.00″W) were located in Córdoba; the holm oak cropping systems [reforestation (37°39′38.21″N, 6°01′08.21″W) and dehesa (37°42′35.47″N, 5°59′56.10″W)] were located in Sevilla; the organic olive orchard (37°01′10.40″N, 4°41′29.87″W) was located in Málaga.

In each cropping system with trees (all except for the sunflower plantation), four sampling points in each experimental field were placed in a square at a distance of 100 m, as shown in Fig. 1. At each of the four sampling points, the following samples were collected: (1) Soil sample: 100 g of soil was collected from 10 cm of the upper soil layer located beneath the canopy at the North, East, West, and South of a tree. The soil samples were placed individually in sterile plastic bags. (2) Leaves from the tree: six leaves each from the North, East, West, and South of the tree were collected and placed individually in sterile plastic bags. (3) Leaves from three weeds located near that tree were sampled and placed individually in sterile paper bags. These samples were collected once per season (spring, summer, autumn and winter) during one year (2010). In the sunflower plantation, four sunflower plants at sampling points in each experimental field were placed in a square at a distance of 100 m, and one soil sample was selected near each plant. Six leaves from each sunflower plant were collected at the following plant developmental stages: the hypocotyl stage (A1), the late floral button stage (E4), and the end of flowering (MO). The tools used for the soil and leaf sampling were surface sterilized with 70% ethanol between each sampling.

The leaves collected in the field were placed individually in sterile paper bags and transported to the laboratory, where each side of the leaf was "printed" onto 1/3 of a section of a 90-mm diameter Petri dish containing a glucose Sabouraud-chloramphenicol agar medium and two selective media for *Metarhizium* (1% glucose, 1%

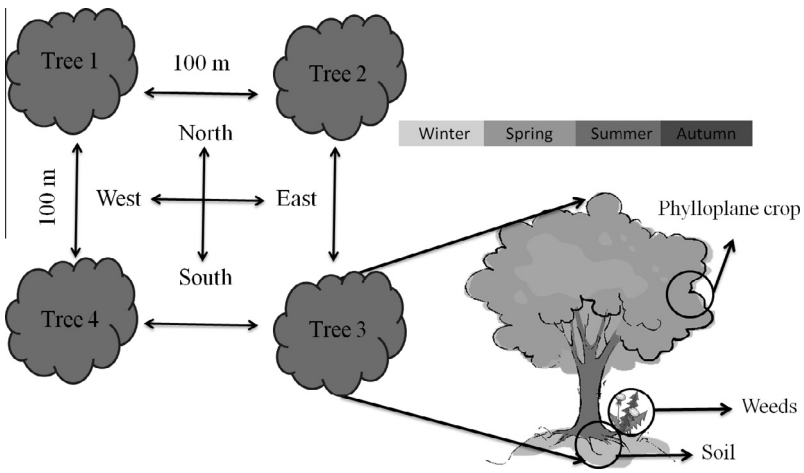

Fig. 1. The sampling points of each cropping system. Each tree was separated from one another by 100 m. Soil, crop leaves, and leaves from three weeds were collected at different orientations (North, East, West, South) during all 4 seasons [Winter, Spring, Summer and Autumn] during one year.