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Metabolic profiling reveals a functional succession of active fungi during the decay of Mediterranean plant litter

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We evaluated the relationship between the potential activity shown by pure fungal isolates (*in vitro*) and their ecological role in the field. Fungal species frequency and occurrence during trophic succession on a natural resource were studied and used as a tool to gain a better understanding of the metabolic profiles obtained *in vitro*, thus permitting clarification of the role played by some of the key fungal species involved in the decomposition process within a Mediterranean natural ecosystem. Litter bags containing pure (homogeneous) and mixed leaves of low maquis plant species were incubated for one year in a field experiment. Functional diversity of a representative pool of fungal communities was analysed and compared using a "phenotype microarray" technique. Taking as its basis the screening of metabolic profiles using culturable isolates, this investigation showed that there was a high potentiality of functional redundancy between low, intermediate and high frequency taxa, and potential niche overlap during the different phases that occur during leaf litter decomposition. The early stages of decomposition appeared to be characterized by the presence of rare taxa ("Low" species), with a significantly higher functional potential, especially for sugar compounds. Conversely, the later phases of decomposition appeared to be characterized by species of intermediate frequency, while rare taxa at these stages seem to lose their dominant role. The importance of the different groups of substrates found across the decomposition phases also represented a key factor. For example, redundancy in the utilization of N-related compounds indicated a high potential for overlapping between species, especially in the early phases of the decomposition process, suggesting that the capacity for interspecific competition on some substrates can be considerable, particularly at the start of substrate exploitation.

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1. Introduction

Fungi possess a range of important ecological functions associated with nutrients and carbon cycling processes in leaf litter and soil. Their contribution to the total respiration of soil organisms can exceed 90% (Kjøller and Struwe, 1982). Gourbière and Gourbière (2002) studied the persistence and extinction of a fungal species colonizing natural resource units and showed that the particular biological traits of individuals/species alone are not fully responsible for fungal successions, but instead that biological patterns and processes play a decisive role in the way in which species are assembled in both time and space, a characteristic shared by plant communities which has been recognized for a long time (Watt, 1947). Functional diversity among fungal species that colonize resource units can represent a key factor in the formation of communities and in their establishment in the natural environment, but the study of the same suffers from rather limiting sampling techniques.

The traditional methodology employed for the characterization of microfungi assemblages is direct observation based on the appearance of sporulating structures on the substrate (Fryar, 2002). The presence of fungi on a particular substrate is dependent on the appearance of the fruiting structures, and these often appear only sporadically. If they do not appear or are somehow hidden from sight, it could be that the fungi are in the vegetative state. The mycologist has to be actively observing through the microscope or actually present at the colonized site when fruiting structures appear. It is well known that such structures can be highly ephemeral, and therefore evade being observed. Identification of *in vitro* isolates obtained by using substrate washing techniques can detect viable but dormant species (Ponge, 1991), and only culturable organisms can be identified. Genomic methods are powerful but suffer from the drawback of potential incomplete extraction of

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DNA (Muyzer, 1998), and there is a lack of diagnostic sequences in databases for leaf litter conidial fungi (Rajala et al., 2011). In addition, DNA-based techniques yield no information on active species and functional traits. Recently, Rajala et al. (2011) investigated the changing community of metabolically active fungi during the decomposition of felled *Picea abies* logs by directly extracting and barcode sequencing precursor rRNA. They also discussed the use of RNA as a marker of metabolically active fungi. Apart from the information gained from the highly technological approaches adopted in some studies, the link between specific functional traits and actively growing conidial fungi in leaf litter decomposition dynamics is still considerably lacking, or at least poorly understood.

Assessment of fungal functional diversity has previously concentrated on evaluating the ability of selected fungi to utilize simple sugars, cellulose, or lignin (Kjøller and Struwe, 1992; Lindahl and Finlay, 2006; Promputtha et al., 2010; Rinkes et al., 2011; Koukol and Baldrian, 2012). Whilst obtaining such information permits the assignment of fungi into broad physiological groupings, this approach does not provide the tools necessary for the evaluation of large-scale patterns in fungal functional diversity, since all systems contain fungi capable of decomposing the three aforementioned major classes of plant compounds (Dobranic and Zak, 1999).

This shortcoming can be overcome by using the Phenotype MicroArray™ system (Bochner, 1988; Bochner et al., 2001) which allows for the testing of microorganisms against different carbon sources. Setting out from the Garland and Mills method (1991) for the functional characterization of microbial communities, Buyer et al. (2001) developed a suitable method for the characterization of fungal communities by outlining the metabolic profiles of mixed inocula. The substrate utilization assay, based on 96-well plates, is simple, straightforward, and suitable for fungal communities originating from different matrices.

The carbon source Phenotype Microarrays were used to detect the metabolic profiles of single fungal isolates (Kubicek et al., 2003; Tanzer et al., 2003; Deacon et al., 2006; Druzhinina et al., 2006). This approach is important because it represents a starting point for uncovering the ecological niche of a fungus (Atanasova and Druzhinina, 2010).

Several taxonomic studies carried out in the Mediterranean region have been directed to the study of fungal communities that colonize single plant species (Mulas et al., 1990, 1995; Pasqualetti et al., 1999; Tempesta et al., 2003, 2005) or mixed litter containing a selection of plant species (Vardavakis, 1998; Markis and Diamantoglou, 1990; Lunghini and Quadraccia, 1990; Lunghini, 1994; Zucconi et al., 1997; Pinzari and Lunghini, 2002), but to our knowledge no papers are currently extant dealing with the metabolic fingerprint of saprophytic fungi originating from Mediterranean regions. Deacon et al. (2006) studied the functional diversity of soil fungi in a grassland ecosystem by applying an invaluable approach which, if applied to Mediterranean leaf litter and soil, could improve our knowledge and understanding of the dependency of decomposition rates on the number, frequency and functionality of fungal species in the Mediterranean climate.

The aim of the study was to evaluate the relationship between the potential activity shown by pure fungal isolates (*in vitro*) and their ecological role in the field. Fungal frequency and occurrence during the trophic succession on the natural resource were studied and used as a tool to gain a better understanding the metabolic profiles obtained *in vitro*, thus permitting a clarification of the role played by some of the key fungal species involved in the decomposition process within a Mediterranean natural ecosystem.

This experimental approach was devised to estimate the possible role of fungal succession in decomposition processes and to determine whether or not: (1) soil fungal community functional diversity actually changes during the natural succession on the trophic resource; (2) fungal appearance and disappearance during a decay chronosequence is driven by a different ability of the species to degrade organic compounds.

2. Material and methods

2.1. Study site

The study was carried out in the evergreen Mediterranean shrubland located in the Castel Volturno Nature Reserve, southern Italy (Latitude 40° 93′, Longitude, 14° 01′), which is characterized by the evergreen sclerophyllous-shrub cover of the low maquis and is mainly constituted by *Phillyrea angustifolia* L., *Pistacia lentiscus* L. and *Quercus ilex* L., and the mesophyllous shrubs *Cistus incanus* L. and *Cistus salvifolius* L., which are early colonizers following disturbance (De Marco et al., 2008).

2.2. Experimental design

We selected three widespread species of sclerophylls (*P. angustifolia* L., *P. lentiscus* L. and *Q. ilex* L.), together with mesophyll *Cistus* sp. pl. Litter bags containing pure (homogeneous) or mixed leaves of low maquis plant species were incubated during a yearlong field experiment, in accordance with De Marco et al. (2011). Samples of all litter bag types were incubated at four field sites under the canopy of the pre-selected plant species commencing in October 10, 2006. We examined 96 samples, deriving from 4 field sites; there were 3 sampling intervals and 3 repetitions of litter bag types. We sampled in January, 2011 (92 days, Day 92), April (188 days, Day 188), November (403 days, Day 403), and twenty leaves of each plant species from each litter bag were investigated by the Direct Observation method after 15 days of incubation in a sterile humid chamber at 20 °C (Cannon and Sutton, 2004). The upper surfaces of ten of the twenty leaves were examined, as well as the lower surfaces of the ten remaining. This procedure was not applied to the *Cistus* leaves, since when they are dry it is impossible to distinguish between the upper and lower surfaces.

A total of 3820 leaves were examined; 118 microfungal species were identified, based on morphology (such as peculiarities of conidiomata and conidia, as well as the conidiogenesis), with 105 species belonging to anamorphic fungi and the remaining 13 species belonging to Ascomycota. Some species, which covered a range of different frequencies, were isolated directly from the leaves, with the help of home-made glass needles, from fungal structures (mainly mycelium, sclerotia and fruiting bodies) visible at a stereomicroscope, and cultured in Potato-Dextrose Agar (PDA) plus streptomycin sulphate (100 γ/mL of substrate). Some strains required several passages to be separated from other fungal species.

2.3. Selection of species representative of the fungal communities detected

Indicator Species Analysis (ISA) (Dufrêne and Legendre, 1997) was used on the matrix containing the identified fungal species and their occurrence, to determine how closely each species (using species frequency) was associated with these three categories: 1) leaf litter species, 2) monospecific or mixed, 3) sampling times. The ISA and Monte Carlo test were performed by means of PC-ORD 5.33 (McCune and Mefford, 2006), with 15,999 permutations being used for the Monte Carlo test. Of the 118 species recorded, 13 anamorphic fungi (Table 1) isolated on agar were analysed and compared by means of Phenotype Microarrays (Atanasova and Druzhinina, 2010). Ten of these (*Acremonium* sp., *Anungitea fragilis*, *Beltrania*

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