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Automated image-based analysis of spatio-temporal fungal dynamics

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ABSTRACT

Due to their ability to grow in complex environments, fungi play an important role in most ecosystems and have for that reason been the subject of numerous studies. Some of the main obstacles to the study of fungal growth are the heterogeneity of growth environments and the limited scope of laboratory experiments.

Given the increasing availability of image capturing techniques, a new approach lies in image analysis. Most previous image analysis studies involve manual labelling of the fungal network, tracking of individual hyphae, or invasive techniques that do not allow for tracking the evolution of the entire fungal network. In response, this work presents a highly versatile tool combining image analysis and graph theory to monitor fungal growth through time and space for different fungal species and image resolutions. In addition, a new experimental set-up is presented that allows for a functional description of fungal growth dynamics and a quantitative mutual comparison of different growth behaviors.

The presented method is completely automated and facilitates the extraction of the most studied fungal growth features such as the total length of the mycelium, the area of the mycelium and the fractal dimension. The compactness of the fungal network can also be monitored over time by computing measures such as the number of tips, the node degree and the number of nodes. Finally, the average growth angle and the internodal length can be extracted to study the morphology of the fungi. In summary, the introduced method offers an updated and broader alternative to classical and narrowly focused approaches, thus opening new avenues of investigation in the field of mycology.

1. Introduction

Fungi are present in and affect most natural, agricultural and urban environments. In forests, fungi are the primary decomposers of organic matter (Krivtsov et al., 2006), where they also form mycorrhizal associations with tree roots allowing for an effective distribution of nutrients across long distances (Boddy et al., 1999; Dickson and Kolesik, 1999). In addition, pharmaceutical and food industries benefit from fungi considerably, as they are used to create products ranging from alcohol and bread to industrial enzymes and antibiotics (Grimm et al., 2005). Furthermore, fungi are used in bioremediation to fight plant pathogens affecting crops, such as insects and other fungi (Weinzierl and Henn, 1991; Alvindia and Natsuaki, 2008). Nevertheless, fungi are also responsible for wood decay (Schwarze, 2007); therefore, wooden material (Wadsö et al., 2013) and plants (Henkel et al., 2012) are subject to their attack, causing economic losses in construction and agricultural industries.

For all these reasons, fungi have been studied extensively over the years (Boswell et al., 2003; Gadd et al., 2007; Schwarze, 2007). They are characterized by a unique structure, which allows for an efficient internal transportation of nutrients and a rapid expansion in a multitude of different environments, even in extreme conditions (Magan, 2007). Fungi are composed of a vegetative body, called the mycelium, formed by a network of cylindrical thread-like structures, referred to as hyphae. Through these hyphae, nutrients are absorbed and distributed. Fungal growth takes place at the tips of the hyphae (Edelstein, 1982), which are referred to as apices. This gain in biomass can be observed when existing hyphae elongate or when new tips emerge along an existing hypha, a process known as branching (Edelstein, 1982). Supplemental Video S1 illustrates the growth of a fungus

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(*Rhizoctonia solani*) over a period of 75 h and clearly shows the elongation and branching processes. As hyphae grow and explore their surroundings, they may encounter other hyphae, which sometimes leads to fusion of hyphae, called anastomosis, thereby increasing the efficiency of the nutrient cycle and altering the shape of the network (Simonin et al., 2012).

There have been many efforts spent to understand the dynamics of fungal growth. Some researchers focus on local features, like the diffusion of nutrients within the fungal network (Tlalka et al., 2007) or the way fungal tips extend (Bartnicki-Garcia et al., 1995). Others consider the mycelium as a single entity and study its macroscopic characteristics, such as its density and the total area it covers (Davidson, 1998; Falconer et al., 2005). Alternatively, a third group of researchers focuses on the individual hyphae (Boswell, 2008; Carver and Boswell, 2008; Hopkins and Boswell, 2012), as such studying the fungi from a mesoscopic perspective.

A major concern with these different approaches is the evaluation of the results. Laboratory experiments are expensive and time consuming (Fricker et al., 2009), and typically lead to results that depend on the specific experimental conditions and that make it difficult to compare with other experimental set-ups (Jacobs et al., 2002). Furthermore, some of the *in vitro* methods are destructive and do not allow for tracking the growth process through time (Wadsö, 1997), implying that experiments have to be repeated in order to account for the natural variability among individuals. As a consequence of these limitations, many researchers use data from literature to evaluate their experiments (Meškauskas et al., 2004; Boswell, 2008; Fuhr et al., 2012), mostly in a qualitative way. Unfortunately, the availability of this kind of data is limited and it is usually decades old (Trinci, 1974; Hutchinson et al., 1980), as such constraining studies to a few growth scenarios, environmental conditions and/or fungal species.

Given the increasing availability of image-capturing techniques (see Falconer et al., 2010), an interesting alternative is the use of image analysis. Capturing images is easy and does not require expensive equipment. Image analysis has already been used for the study of fungal growth, but most studies involve manual labelling of the fungal network (Trinci, 1974; Heaton et al., 2010), tracking of individual hyphae (Bolton and Boddy, 1993; Diéguez-Uribeondo et al., 2004) or invasive techniques (Barry et al., 2009), which do not allow for tracking the network through time. These techniques are also time consuming and tedious.

In response, this work presents an automated procedure combining image analysis and graph theory to track fungal growth through space and time. The only experimental input needed is a time series of images of a developing fungal network. Hence, direct interaction with samples during the growth phase is not necessary and deterioration of the samples is therefore avoided. The images are automatically processed and transformed into simpler binary representations of the entire fungal network, rather than restricting to a relatively small, unrepresentative part of the network. In this way, the issues of manual labelling, restriction to local scales, and destructive techniques are overcome by this method.

These binary images are composed of thin connected lines and can therefore be easily mapped to mathematical graphs. Using properties of these graphs, we are able to track some of the most relevant mesoscopic fungal growth characteristics through time. In order to study the growth efficiency of fungi, we track measures such as the evolution of the number of tips, the total length of the mycelium (Trinci, 1974; Prosser and Trinci, 1979; Boswell, 2008) and the fractal dimension, which indicates the ability of the fungus to fill the space available (Bolton and Boddy, 1993; Boddy et al., 1999; Blackledge and Barry, 2011). Since the morphology of fungal networks is crucial for the productivity of industrial processes involving fungi (Barry et al., 2009), we also quantify some morphological characteristics, such as the growth angle and the mean internodal length. Hence, the output of the proposed method includes the most commonly studied topological measures of fungal networks. In addition, an innovative experimental set-up is presented in order to test the image analysis procedure. These experiments result in time series of five different fungal species from five different genera, as such allowing for a mutual comparison of their growth dynamics.

2. Materials and methods

2.1. Selected organisms

In this study, we aim at analyzing the dynamics of fungal growth. For this purpose, five different fungal species were selected based on their growth characteristics and their impact on human health and industrial processes. Mother cultures of *Coniophora puteana* (a fungus causing brown rot commonly found on timbers and other wood construction materials (Green and Highley, 1997; Viitanen et al., 2010)), *Phanerochaete velutina* (plant pathogen often used in image analysis studies (Heaton et al., 2010; Obara et al., 2012)), *Trichoderma viride* (fungus used in commercial production Barry and Williams, 2011 and bioremedation Joshi et al., 2011), *Rhizoctonia solani* (plant pathogen with a vast number of hosts worldwide Bailey et al., 2000; Jacobs et al., 2002; Boswell et al., 2003) and *Penicillium lilacinum* (used in the pharmaceutical industries as a source of antibiotics Geng and Yuan, 2010) were maintained on 4% malt agar (2% agar Bacteriological No. 1 (Oxoid), 4% malt extract) for one week at 23 °C ± 2 °C and 65% ± 5% relative humidity in a temperature-controlled room.

2.2. Experimental microcosms

Since we attempt to replicate a scenario for fungal growth with a limited number of external factors, the substrate was limited to a maximum of 0.3 g malt agar in the entire Petri dish with a diameter of 90 mm. In order to maintain the environmental conditions needed for sustaining fungal growth, we added a droplet of substrate in the center of a Petri dish and some additional droplets along the edge of the disk, as shown in Fig. 1. An inoculum of size 6 mm by 3 mm, cut from the periphery of the mother culture, was placed on top of the central droplet. Since images obtained with classical devices are two-dimensional, we must limit the space inside the Petri dish in such a way that its vertical dimension may be neglected. This was achieved by placing the sample on the Petri dish lid and then sealing it with the bottom half of the Petri dish (Fig. 1). In this way, the surface of the lid and the bottom were almost in contact, thereby limiting the maximal growing height to approximately 0.6 mm.

The samples were kept during 24 h in a conditioned cabinet, after which they were transported to another dark cabinet with similar environmental conditions (23 °C ± 2°C and 65% ± 5% relative humidity) and positioned on a flatbed scanner (Epson Perfection V750-M Pro Scanner). Images were captured automatically using VueScan (VueScan 9.4, Hamrick Software, USA) every 30 min for 75 h, as such producing a total of 150 images per sample.

The images have a resolution of 1200 dpi. A maximum of six samples could be scanned simultaneously, resulting in images of $10,000 \times 14,040$ pixels. These images were cropped automatically to focus on the growth area of interest. The final images have dimensions of 2125×2125 pixels per sample, corresponding to approximately 4 cm × 4 cm and representing the central area of the Petri dish containing the initial inoculum.

In order to prevent the presence of possible erroneous information, the droplets of agar and the initial inoculum were removed