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Inoculation order of nectar-borne yeasts opens a door for transient species and changes nectar rewarded to pollinators

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ABSTRACT

Nectar-borne yeast communities are species poor assemblages compring is a few specialized taxa (Saccharomycotina) and many transient species. Short flower lifetimes and harsh environmental conditions impose an enormous pressure on nectar-colonizers, which try to overcome these challenges through fast multiplication and osmotolerance. Since these traits are exclusively known for ascomycetes, the origin of multi-species communities is still poorly understood.

We conducted field and laboratory experiments to analyze the competition between autochthonous pollinator-borne and transient yeast species in nectar. Subsequently we analyzed the impact of microbial growth on the environment.

Our results endorse theories on priority effects and show that yeast incidences in natural flowers, cell densities in microcosms and the environmental impact strongly depend on the inoculation order of the respective yeast species. Transient species are more frequent in flowers visited only once, while specialists require several flower visits to establish common population structures most probably through tough inner-floral competition.

1. Introduction

Nectar dwelling microorganisms provide a good model system for testing hypotheses on community ecology and species interactions. Communities of nectar-borne yeast are species-poor, and nectar open to pollinator-visits bear only a few species per flower (e.g. Pozo et al., 2011). The meta-community regularly isolated from flowers is also very small: a narrow group of highly specialized species from fermenting osmophilous yeasts (Saccharomycotina) are the most prominent members (Lachance, 2006) and are regularly isolated in high numbers or cell densities (Herrera et al., 2009). In addition, a larger number of transient species primarily from soil (Yurkov et al., 2012) or plant-related substrata, such as leaf surfaces, plant tissues, and fruits are regularly isolated from nectar (Mittelbach et al., 2015). They are commonly not regarded as autochthonous in this habitat, since most of these species only occur in small cell densities (measured as Colony Forming Units, CFU) and are not reported to be capable of growth in high sugar concentrations typically found in nectar (Brysch-Herzberg, 2004).

It has been hypothesized, that transient and specialized species have fundamentally different requirements to the floral niche (Vannette et al., 2013). While transient ones are regarded to be accidentally inoculated into nectars and, therefore, do not necessarily rely on maintenance in this niche, specialized species require repeated vectoring to new flowers to escape the dead-end of floral senescence. Impacts on the floral habitat by yeast community growth in nectar, such as alterations of chemical composition or the emission of volatiles may directly affect flower attractiveness to biotic pollinators, thereby influencing the number of visits and vectoring events for nectar-borne microorganisms. As a consequence, the environmental footprint of specialized species is expected to be substantially smaller than that of transient species, or adapted, either beneficial or neutral to pollination-services (Herrera et al., 2013).

While the impact of transient species on pollinators, pollination, or plant fecundity has not been studied to our knowledge, specialized yeast species are reported to be either beneficial to

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pollinator visitation rates (Herrera et al., 2013) or at least neutral to nectar removal (Vannette et al., 2013) and pollinator choices (Schaeffer et al., 2014). This is somewhat remarkable, since osmophilous ascomycetes are well known for their fermentation abilities (Ruyters et al., 2014) and the accompanied acidification of the environment does not conform to preferences of pollinators (Vannette et al., 2013). Most transient species belong to the basidiomycetes, and are members of Tremellomycetes (Agaricomycotina) and Microbotryomycetes (Pucciniomycotina). They lack the ability to ferment sugars but effectively consume a broad spectrum of carbon sources (polysaccharides, polyoles, acids, phenols) and produce extracellular polysaccharides (e.g. capsules), glycoproteins, enzymes and volatiles (Kurtzman et al., 2011).

Sugar composition of nectars is subjected to pollination syndromes, but mostly consists of three main components: sucrose, glucose, and fructose (Baker and Baker, 1983). Preferences for sugar compositions by yeast species are species specific as shown in assimilation profiles (Kurtzman et al., 2011). Moreover, nectar-dwelling microorganisms, especially ascomycetes commonly alter sugar composition (Lievens et al., 2014). In natural flowers, unidentified yeast communities resulted in a relative decline of sucrose and an increasing fructose percentage (Herrera et al., 2008).

Studies to date suggest that the composition of nectar-borne communities and, therefore, the environmental impact on nectar and pollination highly depends on the particular inoculated species (species identity) as well as the order of species inoculation. Generally, a rapid growth is advantageous, but the development of all species is likely to be suppressed when a competitor has been inoculated previously. This so called “priority effect” depends on the species ability to compete for environmental resources (Peay et al., 2012), its adaptation to severe conditions (Vannette and Fukami, 2013) and physiological properties (Crowther et al., 2014), and environmental shifts, such as temperature variability (Tucker and Fukami, 2014). Although, this framework has been successfully applied multiple times to this habitat, yeast species investigated to date were either typical nectar-borne or insect-related ascomycetes from the Saccharomycotina. Transient species, which have lately been acknowledged as members of nectar-borne communities (Mittelbach et al., 2015) have never been studied in this context, although they differ substantially in their functional traits, i.e. growth rates, assimilation of carbon sources, cardinal temperatures (Kurtzman et al., 2011).

In this study we analyze the effects of multiple inoculation events by nectar-probing bees on the community structure of nectar dwelling yeast in flowers of *Echium plantagineum*. A subset of frequent specialist and generalist yeast species is then used in a popular framework of inoculation experiments to understand mechanisms of community development. Alteration of chemical composition of media is analyzed subsequently to assess the degree of possible environmental impact of different yeast species and yeast species assemblages.

2. Materials & methods

2.1. Experimental plots

Approximately 60 individual plants of *E. plantagineum* were grown from seeds and planted prior to flowering in an experimental field of the botanical garden of the University of Bonn in May 2012. Plants were arranged in grids of 60 cm × 60 cm. During peak anthesis, the major inflorescences of 20 randomly chosen individuals were used in the experiments, while the remaining plants were left for regular pollinator visitation.

2.2. Experimental setup of pollinator experiment

Prior to each experimental trial, all open flowers from focal plants were removed and inflorescences were covered carefully with sterile nets to avoid pollinator visits and to keep floral nectar free of yeasts (Hilkenbach, 1911). After 24 h the experiments were started by removing the covers one by one. We allowed pollinating insects to visit the flowers either only once (single visits), for 24 h, or for 48 h. In the case of single visits, we identified the visiting species, recorded the number of visits of this particular individual at the floral inflorescence, and marked the flowers for further recognition. After the respective visitation time, inflorescences were covered again to allow the development of microbial communities. After 72 h, close to natural senescence of flowers of *E. plantagineum*, flowers were harvested.

2.3. Yeast isolations

Focal flowers were removed carefully from inflorescences, stored in sterile plastic bags, and processed in the laboratory within 1 h. The whole nectar contents of each flower were diluted in 100 μl of sterile tap water and distributed on the surface of YM agar plates (Yeast Media: 0.3% w/v Yeast extract, 0.5% w/v Peptone, 0.3% w/v Malt extract, 1% w/v Glucose, 1% w/v Fructose and 1% w/v Sucrose, 2% w/v Agar). Sealed plates were then stored at room temperature and regularly monitored to avoid the loss of yeast colonies due to rapid mould growth. After 6 d colonies were differentiated into macro-morphological types using dissection microscopy, counted, and single representatives of each colony type per plate were transferred into pure culture. DNA was isolated from 3 to 4 d old cultures using a chlorophorm-phenol technique and species identification was performed by sequencing of rDNA regions as described by Yurkov et al. (2012). DNA fragments were amplified by PCR using the primers ITS1f, NL1 and NL4. Sequencing of resulting PCR fragments was performed with the same primers.

2.4. Yeast identification

Sequence chromatogram trace files were manually edited using Sequencher 5.0 (Gene Codes Corp., USA) to assure high quality. We identified the isolated strains on the basis of the phylogenetic placement using Maximum-Likelihood analyses (raxML: Silvestro and Michalak, 2011) of a cured (Gblocks: Castresana, 2000) alignment (MAFFT: Katoh and Standley, 2013) (Supplementary Fig. 1). One representative sequence per phylogenetic clade was used to search for the best matching sequences in the NCBI GenBank using the nBLAST algorithm (Altschul et al., 1990).

2.5. Setup of competition experiments

To understand better interactions of yeast species observed in the field study, we simulated competition growth experiments under controlled lab conditions following the protocol by Vannette and Fukami (2013). In our experiment we used four yeasts frequently isolated from flowers, namely two nectar-borne specialist ascomycetes *Metschnikowia reukaufii* (MOM_356 = DSM 100740) and *Candida rancensis* (MOM_447 = DSM 100742), and two transient basidiomycetes *Udeniomyces pannonicus* (MOM_362 = DSM 100741) and *Cryptococcus victoriae* (MOM_325 = DSM 29088). All used strains were isolated in this study from floral nectar of *E. plantagineum* and are deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ).

In short, the experiment was performed as follows. We created artificial nectar environments consisting of sugars and amino acids approximating amounts and composition of natural conditions in *E.*

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