Yeasts in floral nectar of some South African plants: Quantification and associations with pollinator type and sugar concentration

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Abstract

There is increasing evidence that nectarivorous yeasts are an important third player in plant–pollinator mutualisms, but their distribution and ecological effects remain poorly known. Here we provide a survey of the frequency and abundance of yeasts in floral nectar from 40 taxonomically diverse South African plant species, test whether they affect nectar properties, and investigate associations between yeast incidence and pollinator type. Microscopical observations of nectar samples revealed that yeasts are widespread in floral nectar of South African species, as revealed by the high percentage of plants (51.3%) and flowers (43.2%) containing those microbes, and that when present, they can reach high densities (up to $3.6 \times 10^6$ yeast cells/mm$^3$ in *Moraea graminicola*). Further, a significant negative correlation was found between yeast density and sugar content ($R_s = -0.463, P = 0.039$) and yeast density and nectar concentration ($R_s = -0.470, P = 0.037$) in a *Watsonia* species. Interestingly, variation in yeast incidence among plant species was related to differences in pollinator type, in such a way that the plant species pollinated by birds showed the highest proportion of plants and flowers with yeasts, while those visited only by Hymenoptera showed the lowest values. Our study confirms the ubiquity of nectarivorous yeasts in plant communities and identifies novel ways of approaching the study of nectar characteristics and exciting new perspectives on the role of yeasts in plant–pollinator relationships.

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1. Introduction

Integration of separate scientific disciplines is necessary for better understanding of the complex processes occurring in nature (Bechtel, 1993; Fischer et al., 2003; Relyea and Hoverman, 2006). These links can be useful in enabling researchers working on a particular topic to address unresolved questions by integrating and using developments in another body of knowledge. Ecology has been traditionally considered as “inherently cross-disciplinary” (Madin et al., 2007), yet a significant lack of integration has been identified in some research areas, highlighting the need for a combined approach to provide a clearer and broader view of the nature of interactions among individuals and species and the consequences of these interactions (e.g. Kearsley and Whitham, 1997; Clarke and Young, 2000; Dahdouh-Guebas and Koedam, 2008; Herrera et al., 2009).

Plant–pollinator interactions have traditionally been assumed to be binary systems in which both partners should obtain a net benefit: reproduction on the one hand (plants) and food resources on the other (pollinator) (Bertin, 1989; Bronstein et al., 2006; Raguso, 2008a). Nevertheless, recent studies have considered three-way interactions linking plants, pollinators and some third party such as herbivores (Herrera, 2000), mycorrhizal fungi (Gange and Smith, 2005; Gehring and Bennet, 2009), or nectarivorous yeasts (Canto et al., 2007, 2008; Herrera et al., 2008, 2009). That yeasts are frequent inhabitants of floral nectar has been well known for more than a century (Boutroux, 1884; Nadson and Krassilnikov, 1927) and there has been an increasing interest among microbiologists in identifying nectarivorous yeasts (Lachance et al., 2001; Herzberg et al., 2002; Brysch-Herzberg, 2004; Manson et al., 2007). However most of these microbiological studies on nectar yeasts have largely been ignored by ecologists, and this persistent lack of integration among the two
research areas has perpetuated significant gaps in our knowledge of the ecology and evolution of plant–pollinator interactions.

Despite the scarcity of published studies, the available data suggest that nectarivorous yeasts, which are transported from flower to flower by floral visitors, are quite widespread in ecologically different plant communities, reaching high densities in floral nectar (Jimbo, 1926; Sandhu and Waraich, 1985; Brysch-Herzberg, 2004; Herrera et al., 2009). However, their effects have only recently begun to be studied. It has been demonstrated, for example, that yeasts can alter nectar characteristics by diminishing drastically nectar sugar concentration and altering nectar sugar profiles, i.e. the proportion of sucrose, glucose and fructose (Canto et al., 2007, 2008; Herrera et al., 2008) entailing a decrease of nectar energetic value from the viewpoint of pollinators. Moreover, Herrera et al. (2009) have also demonstrated an interesting connection between pollinator type and yeast incidence in mountain plants in Southern Spain, a link between pollination ecology and floral nectar microbiology that has remained unexplored until now. Since nectar is an important floral reward offered by plants to pollinators (Simpson and Neff, 1983; Galetto and Bernardello, 2005; Nicolson, 2007) and it plays a decisive role in the establishment of plant–pollinator mutualisms (Pyke et al., 1988; Rathcke, 1992; Johnson et al., 2006; Jersáková et al., 2008; De Vega et al., 2009), nectarivorous yeasts could commonly affect pollination success, pollen flow and plant fitness.

Qualitative and quantitative studies on nectarivorous yeasts have been lacking for nectar-producing plants in southern Africa, one of the world’s biodiversity hotspots. This information may be essential for a better understanding of the distribution of these microbes throughout the world, as well as to corroborate the generality of recent findings for Mediterranean plant species of nectar sugar degradation by nectarivorous yeasts (Canto et al., 2007, 2008; Herrera et al., 2008). Particularly, the study of plant and animal communities in ecologically disparate scenarios is particularly relevant to test the hypothesis that variation among species in yeast incidence is correlated with differences in pollinator composition.

We carried out a study in different South African plant communities. Our specific goals were: (i) to ascertain, by means of microscopic observations, the frequency of occurrence (proportion of flowers and plants containing yeast-infected nectar) and abundance (cell density in nectar samples) of yeasts in the floral nectar of many taxonomically different plant species from ecologically different locations; (ii) to test previous findings showing changes in some nectar features with increasing yeast density; and (iii) to examine possible relationships between yeast incidence and pollinator composition.

2. Materials and methods

2.1. Study species and study sites

Our survey includes nectar samples from 40 plant species belonging to 19 families. The taxonomic distribution among families of species sampled as well as the complete list of the species surveyed is shown in Fig. 1. Floral nectar collection was carried out at a number of localities in KwaZulu-Natal province of South Africa, including the Blinkwater Nature Reserve, Garden Castle (Ukhahlamba-Drakensberg Park), Mount Gilboa in the Karkloof Range, Sani Pass below the South African border post, Umkomaas Valley, Vernon Crookes Nature Reserve, and Wahoonga farm. Sites differed in ecological characteristics including elevation, soil, and type of vegetation (Table 1).

2.2. Methods

2.2.1. Yeast survey

Single flowers or inflorescences already open and exposed to pollinators were collected in the field, placed in jars filled with water and carried into a cooler to the lab where nectar sampling and microscopic observations were done within the following 24 h after flower collection. Nectar samples from individual flowers (mean±SE=2.2±0.1 flowers per plant) were collected on different flowering individuals of every species (mean±SE=7.9±0.8 plants per species). Yeast presence and abundance were assessed in 635 flower nectar samples, on an average (±SE) of 15.9±1.0 flowers per species.

Nectar was extracted from each sampled flower with a micro-capillary, its volume determined by the length of the column, and then diluted with 30% lactophenol cotton blue solution to facilitate microscopic detection of yeast cells. Yeast concentration (cells/mm³) was estimated under a light microscope with medium magnification (20×–40× objectives) using a Neubauer improved cell counting chamber (Marienfeld, Germany). Although bacteria were also found to be frequent inhabitants of floral nectar, the clearly different yeast morphological features, their bigger size, presence of large vacuoles containing highly refractive corpuscles, evidence of division by budding and our gained expertise allow an unequivocal counting and identification of yeasts (Fig. 2). This level of taxonomic resolution is sufficient for the purposes of this study, and we are confident that microorganisms reported in this study are yeasts in all cases.

2.2.2. Impact of yeasts on nectar characteristics

In order to determine if there is a correlation between nectar characteristics and yeast density a more-detailed study was carried out in the species Watsonia pillansii (Iridaceae). Seven flowering individual plants naturally exposed to pollinators were collected in the field and carried within a cooler to the lab where flowers were immediately analyzed. A random sample of N=21 flowers (sampled flowers were already open, and thus exposed to pollinators) was collected from different plants (N=3 flowers/plant). In each flower 1 µL of nectar was extracted with a capillary tube: a 0.5 µL aliquot was used to estimate yeast density as explained above, and the remaining 0.5 µL was deposited on a low-volume hand refractometer (Bellingham & Stanley Ltd, Tunbridge Wells, UK) to measure its sugar concentration expressed as percentage sucrose equivalent (grams of sucrose per 100 g of solution). As a proxy to estimate the potential energetic reward available to consumers on a per-flower basis, total sugar content of nectar samples was calculated following Galetto and Bernardello (2005) by converting nectar concentration to milligrams of sucrose per 100 µL of solution. Spearman’s correlations
were used to investigate the relationships between yeast cell density and the concentration and total amount of sugar per flower.

2.2.3. Yeast incidence and pollinator composition

We also checked for a possible relationship between yeast incidence and pollinator composition in a subsample of 23 species. For that purpose, information on the main group of pollinators (Hymenoptera, Diptera, Lepidoptera and Birds) visiting each species was based on direct observations by one of us (S. D. J.). Pollinator assessments were done during different years, mainly in the same places where nectar collections were done. Because data could not be normalized, a Kruskal–Wallis test was used to establish the significance of rank differences in cell density, percentage of plants with yeasts and percentage of flowers with yeasts among plants with different pollination systems.

| Population                          | Coordinates         | Elevation (m) | Geology | Vegetation  |
|-------------------------------------|---------------------|---------------|---------|-------------|
| Blinkwater Nature Reserve           | 29°14'10.08"S 30°27'15.68"E | 1472          | Dolerite | Grassland   |
| Garden Castle (Ukhahlamba-Drakensberg Park) | 29°44'49.83"S 29°12'36.17"E | 1823          | Sandstone | Grassland   |
| Mount Gilboa                        | 29°16'58.24"S 30°17'31.93"E | 1528          | Sandstone | Grassland   |
| Sani Pass                           | 29°37'20.06"S 29°23'34.65"E | 1762          | Basalt   | Grassland   |
| Umkomaas Valley                     | 29°59'15.62"S 30°14'51.45"E | 572           | Shale    | Thicket     |
| Vernon Crookes Nature Reserve       | 30°15'56.67"S 30°36'37.27"E | 463           | Sandstone | Grassland   |
| Wahroonga farm                      | 29°36'28.53"S 30°8'0.75"E | 1466          | Dolerite | Grassland   |

Fig. 1. Phylogenetic relationships of the plant species sampled for this survey, showing the main pollinator type when data available.
A previous study found indirect evidence suggestive of a phylogenetic component underlying the correlation between yeast incidence and pollinator type (Herrera et al., 2009). We made a preliminary assessment of potential phylogenetic biases in our sample by plotting the distribution of pollinator types across a phylogeny of the 40 species sampled. The phylogeny was constructed using the Phylomatic web tool (http://www.phylodiversity.net/phylomatic/phylomatic.html; last accessed 17 February 2009). Although our data were insufficient for properly investigating this possibility through phylogenetically independent contrast (PICs) analyses similar to those described in Herrera et al. (2009), we found that the different pollination systems were thoroughly intermingled across the different branches of the phylogenetic tree (Fig. 1). We thus deemed reasonable to analyze the relationship between yeast incidence and pollinator type by considering species as statistically independent units (TIP analysis).

3. Results

3.1. Yeast survey

Microscopical observations of nectar samples revealed that nectarivorous yeasts are frequent inhabitants in floral nectar of South African plant species, as revealed by the high percentage of plant individuals (51.3%) and flowers (43.2%) that contained these microbes. A complete list of the species surveyed with information on yeast incidence is available as Supplementary information.

The percentage of nectar samples per species containing yeast cells ranged between 0% and 100% (Fig. 3). Yeast incidence was extremely variable among plant species, from species in which yeasts were never observed ($N=8$) to species with all their sampled flowers containing yeasts in nectar ($N=4$). Considering only species with yeasts, the proportion of

Fig. 3. Frequency distributions of the percentage of nectar samples containing yeast microbial communities from the different plant species.
plants per species that contained yeasts ranged from 10% to 100%, while the proportion of flowers per species that contained yeasts ranged from 4.6% to 100%. Mean yeast density was extremely variable among species, ranging from 0 to 881,062 cells/mm³. The highest estimated yeast density (3,640,333 cells/mm³) was observed in a nectar sample from Moraea graminicola (Iridaceae).

3.2. Impact of yeasts on nectar characteristics

Nectar sugar concentration in *W. pillansii* varied widely, ranging between 8.5 and 25%, with a mean (±SE; this notation will be used hereafter) of 16.6 (±1.1; *N* = 21 flowers). Sugar content ranged between 0.093 and 0.273 mg/µL (0.179 ± 0.058). When among-flower differences in nectar features were correlated with variations in yeast density, both nectar concentration and sugar content showed a significant declining trend with increasing yeast density (Fig. 4). There was a significant negative correlation between yeast density and sugar content (*R*$_s$ = −0.463, *P* = 0.039), and between yeast density and nectar concentration (*R*$_s$ = −0.470, *P* = 0.037).

3.3. Yeast incidence and pollinator type

The phylogenetic relationships of plant species sampled in this survey are shown in Fig. 1. Data on yeast presence and pollinator composition were simultaneously available for 23 plant species belonging to 15 families. This subset of species encompassed the whole range of variation in frequency of yeast cells on flowers (0–100%) and plants (0–100%) found in the whole sampling (40 species), and thus was considered as representative of the survey. Moreover there was no evidence of any major phylogenetic biases or correlations in this sample, since both yeasts and the four main groups of pollinators (Hymenoptera, Lepidoptera, Diptera, and Birds) were observed in different families of monocot and dicot species.

Interspecific differences in yeast incidence were related to variation in pollinator type (Fig. 5). The species pollinated by birds showed the highest proportion of plants and flowers with yeasts, while species visited only by Hymenoptera showed the lowest values. When plant species were separated depending on

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**Fig. 4.** Relationships between nectar concentration (a), and nectar sugar content (b) vs. yeast cell density in nectar samples of *Watsonia pillansii*. Each symbol corresponds to a nectar sample from a single flower. Correlation coefficients (*R*$_s$) and the associated *P*-values were obtained through Spearman’s correlations.

**Fig. 5.** Box plots of (a) percentage of plants with yeasts, (b) percentage of flowers with yeasts, and (c) yeast density, shown separately according to pollinator type. Squares represent means, box plots the standard error and whiskers the standard deviation. *Abbreviations*: H, Hymenoptera; L, Lepidoptera; D, Diptera; and B, Birds.
the two main groups of pollinators (birds against insects) significant differences were found in the percentage of plants ($\chi^2=6.659$, $df=1$, $P=0.009$) and flowers ($\chi^2=4.102$, $df=1$, $P=0.042$) containing yeasts. Although there were no differences in yeast cell density between birds and insects ($\chi^2=1.155$, $df=1$, $P=0.283$), plants pollinated by birds showed the higher yeast density, followed by moth-pollinated species (Fig. 5), than Diptera and Hymenoptera.

4. Discussion

By examining nectar samples from taxonomically different plant families, three main findings emerge from our study. First, our survey has shown that yeast communities are frequent in floral nectar of animal-pollinated South African plants. Second, we have found that at least in one of the species sampled, yeast incidence is correlated with drastic changes in some nectar features. Third, our results are suggestive of an association between pollinator type and yeast incidence. The relevance of these results is discussed below.

Leaving aside studies on cultivated plants growing in artificial environments (e.g. Gilliam, 1975; Gilliam et al., 1983), the frequency of occurrence of yeasts in nectar samples of flowers freely exposed to pollinators in this survey (43%, $N=635$ nectar samples) was remarkably similar to that found in previous studies including plants visited by a diverse pollinator assemblage made in Japan (44%, $N=273$, Jimbo, 1926), Spain, and Mexico (42%, $N=2733$, Herrera et al., 2009). These figures are lower than those found in Denmark and Sweden for wasp-pollinated orchids (57%, $N=46$, Ehlers and Olesen, 1997), India for insect-pollinated plants (68%, $N=342$, Sandhu and Waraich, 1985) or Germany for bumble bee-pollinated plants (72%, $N=195$, Brysch-Herzb, 2004). However, our values for yeast density (up to $3.6 \times 10^5$ cells/mm$^3$) are by far the highest ever reported in nectar samples. Despite its simplicity, yeast density-estimates in nectar samples have only been conducted in two previous studies (Brysch-Herzb, 2004; Herrera et al., 2009). Brysch-Herzb (2004) estimated densities of up to 16,000 cells mm$^3$ in exclusively bumble bee-pollinated plant species, while Herrera et al. (2009) in a survey on 130 plant species in the Mediterranean Basin and Gulf of Mexico suggested that densities of approx. $4 \times 10^5$ cells/mm$^3$ were probably “near an absolute ceiling for yeast cell density in floral nectar under natural conditions”, a suggestion that is clearly superseded by the present data. Yeast growth depends on multiple factors such as sugar concentration, nutrient availability, temperature, pH or the presence of inhibitory substances in nectar (Heard and Fleet, 1988; Viegas et al., 1989; Lourens-Hattingh and Viljoen, 2001; D’Amato et al., 2006; Pigeau et al., 2007). However, it is plausible that another factor not taken into account previously, the yeast cell-size, could also be limiting the maximum densities in natural conditions just for physical reasons. Yeast cell-size varies from one species to another (Barnett et al., 2000), and the species involved in the previous surveys made in Spain and Mexico had consistently larger cell sizes than those observed in this South African survey (De Vega and Herrera, pers. obs.). It is likely that due to simple steric (physical) constraints, yeasts with large cell sizes cannot reach the same high densities per microlitre as the smaller ones can.

Yeast incidence has hardly been taken into account in studies of plant–pollinator interactions; however, recent studies have shown the important role they could be playing in this relationship, changing what has been considered a dual system to an ecological trio. It has been recently established that yeasts alter nectar sugar profiles which, acting in concert with small-scale variation in yeast cell densities, may eventually generate high intra-plant variation in sugar composition (Canto et al., 2007, 2008; Herrera et al., 2008). Our data on \textit{W. pilansii} support and extend the view that variation in yeast density will often be correlated with important changes in nectar sugar concentration, as observed by Herrera et al. (2008) in three Spanish plant species, in such a way that the higher the yeast density, the lower the sugar concentration and energetic value of nectar. A reduction in nectar sugar content after pollination has been repeatedly related to nectar reabsorption as a mechanism allowing the plant to recover part of the energy invested in nectar production (e.g. Búrquez and Corbet, 1991; Koopowitz and Marchant, 1998; Luyt and Johnson, 2002; Nepi et al., 2003; Nepi and Sttpiczynska, 2007). Ideally, such experiments should also include verification that yeasts were not introduced to nectar by hand-pollination. However, whenever plants are freely exposed to pollinators and thus to potential yeast contamination, the role of yeasts in sugar consumption needs to be investigated as an alternative hypothesis for reduced sugar content in nectar after pollination. This new perspective calls for different interpretations of results on pollination and nectar characteristics, and considers yeasts as an alternative source of explanations for understanding processes related to post-pollination nectar changes.

On the basis of choice experiments, several authors have stated that insects (Waller, 1972; Pivnick and McNeil, 1985; Cnaani et al., 2006; Whitney et al., 2008) and birds (Stiles, 1976; Roberts, 1996) prefer nectar rewards with higher sugar concentration than dilute solutions. Since yeast-contaminated flowers offer less energetic resources to pollinators, they could be less visited and thus their reproductive success reduced. Thus, one of the primary functions of secondary compounds in nectar might be as antibiotic agents to limit microbial infection (Adler, 2000). In the only published study known to us on the effects of yeasts on pollinator behaviour, Kevan et al. (1988) found that foraging bees did not discriminate between yeast-contaminated and yeast-free flowers. However, neither yeast content nor nectar characteristics were evaluated in that study. Microbial communities inhabiting floral nectar could affect pollinator services and pollination efficiency not only by reducing rewards, but also in several other ways. Fermenting yeasts produce ethanol (Lin and Tanaka, 2006; Hahn-Hägerdal et al., 2007), and the presence of alcohol in nectar could intoxicate and alter pollinator behaviour as suggested by Ehlers and Olesen (1997) and Wiens et al. (2008). Yeasts can also affect seed set in plants by inhibiting pollen germination (Eisikowitch et al., 1990a,b). Another important aspect of yeasts to take into account is their ability to produce a wide range of scents (e.g. Majdak et al., 2002; Swiegers et al., 2005). To what
extent nectarivorous yeast can affect floral scent and filter potential pollinators through volatile release remains to be determined (Raguso, 2008b).

As in the few previous surveys done throughout the world in which a number of plant species were studied (e.g., Jimbo, 1926; Sandhu and Waraich, 1985; Brysch-Herzberg, 2004; Herrera et al., 2009), we have found some taxa with no yeast and others with extremely high values of yeast incidence. Differences in nectar properties could explain to some extent why some species have yeast-free floral nectars. For example, the presence of antibiotic substances in nectar or high solute concentrations could avoid microorganism growth in some plant species (Rabhé et al., 1995; Paccini and Nepi, 2007; Park and Thornburg, 2009). Another, non-mutually exclusive hypothesis so far untested for a tropical system is the existence of a link between pollinator type and yeast incidence. Previous studies have suggested that pollinators are the vectors distributing yeasts among flowers (e.g. Sandhu and Waraich, 1985; Lachance et al., 2001; Brysch-Herzberg, 2004; Canto et al., 2008; Herrera et al., 2009). However, a connection between nectar contamination by yeasts and pollinator assemblage has remained practically unexplored, and only recently an association has been found for some Mediterranean plants, where a direct correlation was found between the proportion of floral visits by humble bees and yeast incidence (Herrera et al., 2009).

In the present study we have observed that bird pollinators seems to be closely associated with high yeast incidence in our sample of South African plants, since bird-pollinated plants have shown to possess the highest percentages of yeast abundance and cell density. Interestingly in our survey we have included a weird special exception that may prove the rule. *Cytanthus contractus* is a plant species adapted to long-billed malachite sunbirds, but none of the local sunbirds in the study area can reach the nectar. Probably the main reason we found no yeasts in the nectar of this species is that the nectar is never utilized in the study area and the plants are pollinated entirely by bees which only collect pollen. The differences found in our study and in previous surveys suggest that the yeast incidence–pollinator association is context-dependent with a strong biogeographic component that deserves further study. The reason why birds are increasing the frequency and cell density on yeasts in floral nectar remains unknown and needs to be studied in depth.

In summary, our survey has shown that yeasts are frequent inhabitants of floral nectar in South African plants, where they often reach extraordinarily high densities, and confirm that yeast presence in floral nectar seems to be an ecological constant, irrespective of continent and habitat type. Additionally, our data also support recent findings suggesting some changes in nectar features after yeast colonization. As previously pointed out (Pleasant, 1983) our results also confirm that caution should be exercised when attempting to characterize nectar features from samples taken under only fixed environmental conditions or at one point in time. We propose that yeasts should be excluded when analyzing the intrinsic nectar properties of a species, while analysis of the potentially yeast-modified standing crop, on the other hand, will still help to understand the behavioural responses of pollinators in the field.

In conclusion, this and other recent studies open up entirely novel ways of approaching the study of nectar characteristics and introduce exciting perspectives for the study of the role of yeasts in plant–pollinator relationships.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2009.07.016.

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