Flower yeasts obfuscate intrinsic individual variation in nectar sugar concentration in a bumble bee-pollinated plant

Carlos M. Herrera [1] and Conchita Alonso

Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Américo Vespucio 26, E-41092 Sevilla, Spain

[1] Author for correspondence: email herrera@ebd.csic.es, telephone +34 95 4466700

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Abstract

Nectar-dwelling yeasts modulate the ecology and evolution of interactions between plants and pollinators. This paper evaluates the hypothesis that yeasts can reshape intrinsic individual differences in nectar traits. We address the question, Do local genotypes of the nectar yeast specialist *Metschnikowia reukaufii* have the ability of obfuscating intrinsic individual variation among *Helleborus foetidus* plants in nectar sugar concentration ? An array of paired plant–yeast genotypes mimicking a natural field situation was created in the laboratory by inoculating nectar from different plant individuals with different yeast genotypes following a factorial design. Chemical analyses of nectar sugars before and after exposure to yeasts were performed using ion-exchange high performance liquid chromatography. Plant individual, yeast genotype, and their interaction all had strong, significant effects on sucrose concentration of *H. foetidus* nectar. Metabolic activity of yeasts abolished 79% of intrinsic variance among plants in nectar sucrose concentration, considerably reducing the opportunity for selection by pollinators on this important nectar trait. We propose that pollinator selection on nectar traits of plant x yeast combinations will supersede "naïve" selection on plants' intrinsic nectar traits, and that nectar phenotypes found by pollinators will largely represent extended phenotypes of the yeast genotype(s) living in the nectar.

Introduction

Phenotypic differences among conspecifics are a central determinant of the opportunity for selection in natural populations. In the case of plants, however, certain traits can sometimes depend more on the expression of genes belonging to intimately associated fungi than to those of the plants themselves, exemplifying instances of fungal "extended phenotypes" (Dawkins 1982; see, e.g., Dawkins 2004, Hunter 2009, 2018, Hawkes et al. 2021, for discussion and reviews). When fungal modifications of plant phenotypes involve floral features (e.g., production of

pseudoflowers), there can be fitness consequences for individual plants via interference with pollinator behavior and plant reproduction (Batra and Batra 1985, Jennersten 1988, Jennersten and Kwak 1991, Roy 1993, 1996, Gange and Smith 2005, González-Mas et al. 2023). Fungi known so far to account for modifications of floral phenotypes mostly are systemic endophytes, generally parasites or pathogens bearing antagonistic relationships with their hosts. Nevertheless, recognition in recent years of the influence of nectar-dwelling yeasts on the interaction between plants and pollinators through their effects on floral nectar and the pollinators themselves (Herrera et al. 2008, 2013, Schaeffer et al. 2014, 2017, 2019, Vanette and Fukami 2016, 2018, Rering et al. 2018, Pozo et al. 2020, de Vega et al. 2022) motivates the hitherto unexplored hypothesis that intraspecific variation in floral nectar traits might actually reflect, to some variable and yet-unknown degree, variation in extended phenotypes of the nectar-dwelling yeasts.

Applicability of the extended phenotype notion to nectar yeasts requires at least that the following three premises hold true: (1) yeast genotypes differ in physiological competence and ability to transform floral nectar, e.g., via changes in sugar composition or concentration; (2) in natural populations of a given plant, floral nectar of different individuals is inhabited by different yeast genotypes, so that plants might present to pollinators nectars with different features *because* they host different yeast genotypes; and (3) differences among coexisting plants in nectar traits of flowers exposed to natural conditions (i.e., visited by pollinators and colonized by yeasts) depend closely on the yeast genotypes living in their flowers. Premise 1 has been documented for the cosmopolitan nectar yeast *Metschnikowia reukaufii* whenever metabolic differences among genotypes have been looked for (Herrera 2014, Pozo et al. 2015, Dhami et al. 2018). Premise 2 is supported by the finding that coexisting individuals of the bumble bee-pollinated herb *Helleborus foetidus* host distinct *M. reukaufii* genotypes in their floral nectar (Herrera et al. 2014). This widespread floricolous yeast (Brysch-Herzberg 2004) is therefore a promising candidate for

testing the notion that nectar yeasts can profoundly reshape intrinsic patterns of individual variation in nectar traits in local plant populations.

This paper reports an experimental test of premise 3 above for the *H. foetidus–M. reukaufii* system. An array of paired plant–yeast genotypes mimicking a natural field situation was created in the laboratory by inoculating nectar from different plants with cells of different yeast genotypes. Sugar analyses of nectar samples before and after a period of yeast growth will be used to answer the following specific question: Do local genotypes of nectar yeasts collectively have the transformative capacity for reshaping intrinsic individual differences in nectar traits ? Our results revealed that the identity of the nectar yeast genotypes hosted by individual plants can be more important than intrinsic plant traits in shaping the variation in nectar traits encountered by foraging pollinators in natural plant populations, which in turn supports the view that pollinators might actually be interacting with sets of extended phenotypes of the nectar yeasts living in the flowers.

Material and methods

Study system and field sampling

This study focuses on the early-blooming perennial herb *Helleborus foetidus* (Ranunculaceae) and the dominant yeast in its floral nectar, *Metschnikowia reukaufii* (Metschnikowiaceae, Saccharomycetales). *Helleborus foetidus* inflorescences are produced in early winter and flowers are pollinated by bumble bees (Herrera et al. 2001). Flowers produce a sucrose-dominated nectar (Vesprini et al. 1999, Canto et al. 2011) that ordinarily harbors dense populations of *M. reukaufii*, the colonizing inocula of which are brought to flowers by foraging bumble bees (Brysch-Herzberg 2004, Canto et al. 2008). Metabolic activity of these yeasts induce substantial changes in composition and concentration of nectar sugars (Herrera et al. 2008, Canto et al. 2008, 2011).

Field sampling was conducted during February-March 2015 in a large population of *H.*

foetidus in the Sierra de Cazorla, Jaén province, southeastern Spain. Floral nectar for the experiment was obtained from 20 widely spaced *H. foetidus* plants whose inflorescences had been previously bagged at the bud stage to exclude pollinators. For each individual plant, nectar from many flowers was gathered into a single bulk sample and stored at -20ºC until used. Ten *M. reukaufii* strains from the same population were randomly chosen for the experiment from a large collection of local strains gathered from nectar of *H. foetidus* flowers exposed to pollinators.

Experimental and analytical procedures

Prior to the experiment, nectar samples were filter-sterilized using a polyvinylidene difluoride 0.22 µm filter. Genetic distinctness of the experimental *M. reukaufii* strains was verified by fingerprinting them using polymorphic nuclear DNA microsatellite markers (Seino et al. 2013), and will be referred to hereafter as "genotypes".

The experimental layout consisted of a two-way factorial design, with *M. reukaufii* genotype (N = 10 levels) and *H. foetidus* individual (N = 20 levels) as main factors, and two replicates per each genotype x individual combination (N = 381 experimental units in total; a few plants had insufficient nectar for replication). Each experimental unit consisted of a 0.2 mL PCR tube containing 15 µL of virgin, sterile nectar plus 1 µL of *M. reukaufii* cell suspension taken from a fresh culture on standard liquid medium. To assess differences among genotypes in their initial cell densities in the experimental units, two replicated control tubes were also set per yeast genotype at the time of inoculation, each consisting of 15 µL of 10% lactophenol cotton blue fixing solution (LCB hereafter) plus 1 µL of *M. reukaufii* cell suspension. After inoculation, all experimental tubes were held at 25ºC without agitation. Two aliquots were taken from each experimental unit for chemical analyses at 24 h and 48 h after inoculation. These rather short periods were chosen to preclude extensive sugar depletion of nectar (Herrera et al. 2008), which

would have biased comparisons.

Samples of virgin nectar from experimental plants (N = 40, two aliquots per plant) and all nectar samples taken in the course of the experiment (N = 762) were analyzed using ion-exchange high performance liquid chromatography. Analytical procedures and equipment were as described in Herrera et al. (2006) and Canto et al. (2011). Separate estimates of the concentration of the three sugars appearing in the samples (sucrose, fructose, glucose) were obtained by integrating areas under chromatogram peaks, and total sugar concentration was computed by summing partial figures. As previously found for *H. foetidus* nectar in the study area (Herrera et al. 2006, Canto et al. 2011), sucrose was virtually the only sugar in all nectar samples. It accounted on average for 99.2% (interquartile range = 99.4–100%) and 98.9% (interquartile range = 100–100%) of total sugars in virgin (pre-exposure to yeasts) and experimental (post-exposure) nectar samples, respectively. Only the results for sucrose will be considered hereafter.

Data analysis

Due to differences among source cultures, initial cell densities in experimental units differed among yeast genotypes (Chi-square = 28.7, df = 9, P = 0.0007, Kruskal-Wallis rank sum test; range of genotype means = 475–1399 cells/mm^3), and experimental plants differed in the sucrose concentration of virgin nectar ($F_{19,20}$ = 73.8, P = 1.7E-14; plant means range = 53–77% weight-to-weight basis). To evaluate the robustness of experimental results to this initial heterogeneity across experimental units, and to accurately dissect the role of yeast genotypes as agents of variation in nectar sugar across plants, two separate statistical analyses were carried out. In the first, sucrose concentration in the nectar of experimental units after exposure to yeasts was regressed on initial yeast cell density, initial sucrose concentration, and their interaction. In the second analysis, a linear model was fitted to the same response variable (post-exposure sucrose concentration) using plant identity, yeast genotype and their interaction as predictors. Including

time of sample collection (24 or 48 h) as another predictor in the analyses had a negligible, statistically nonsignificant effect on results, and the two groups of data were pooled for the analyses. In addition to estimating main effects and interactions in the two linear models, "relative importance" of each predictor was estimated by decomposition of the total variance (R^2) accounted for by each model (Groemping 2007).

Statistical analyses were carried out using the R environment (R Core Team 2024). The raw data used here are available in Herrera (2025). Linear models were fitted using the lm function in stats package. Adequacy of fitted models was verified using the function check_model of the performance package (Lüdecke et al. 2021). Relative importance of predictors was obtained with function calc.relimp in package relaimpo (Groemping 2006). Adjusted predictions (model-based estimates) of the response variable (sucrose concentration) and marginal means were estimated from fitted models using the predict_response function in the ggeffects package (Lüdecke 2018). The specific "blurring" effect of each yeast genotype on individual variation in nectar sucrose concentration was inversely assessed with the correlation coefficient across plants between initial sucrose concentration in virgin nectar and the model-adjusted, predicted values conditioned on the corresponding yeast genotype.

Results

Initial heterogeneity across experimental units in yeast cell density and nectar sucrose concentration had negligible explanatory effects on variation in sucrose concentration after experimental nectar exposure to yeasts, as revealed by both the regression parameter estimates and the proportion of variance explained by single effects and their interaction (Table 1). Exposure to yeasts, therefore, nearly wiped out interplant differences in sucrose concentrations in 48 h. In contrast, plant and yeast identity, and their interaction, each had strong, statistically significant effects on nectar sucrose concentration after exposure to yeasts (Table 1). The

proportion of variance explained by this model was remarkably high (R^2 = 68.1%) given the large number of samples. The estimated contribution of main effects (plant and yeast identity) and their interaction to the variance explained by the model declined in the direction plant (32%) – plant x yeast (20%) – yeast (15%) (Table 1). Model-adjusted, predicted values of nectar sucrose concentration after exposure to yeasts varied among plants (range = 31–53%, conditioned on strain 1_A_10.2) and yeast strains (range = 40–62%, conditioned on plant CV01), but the strong plant x yeast interaction effect renders these figures only weakly informative (Appendix S1: Figure S1).

The high explanatory value of the yeast x plant interaction was of particular significance in relation to the hypothesis tested here. First, the relative effect of different yeast genotypes on nectar sucrose concentration was remarkably different for different individual plants, as illustrated by extensive criss-crossing of lines in the interaction plot of within-plant standardized values (Figure 1). Second, as a consequence of the strong interaction, the set of paired combinations plant x yeast genotype created a heterogeneous two-dimensional space of post-exposure sucrose concentrations (Appendix S1: Figure S1). And third, yeast genotypes differed widely in their capacity to blur individual variation in nectar sucrose concentration, as shown by the broad range across plants of the correlation coefficient between mean sucrose concentration before and after the experiment (r range = 0.142–0.736, mean = 0.454). The metabolic activity of the yeast genotypes tested obliterated between 46–98% (mean = 79%) of intrinsic variance among plants in nectar sucrose concentration over the experimental period (estimated as $1 - r^2$).

Discussion

Local *M. reukaufii* genotypes blurred intrinsic differences among individuals of *H. foetidus* in nectar sucrose concentration, a trait potentially important for the relationship with pollinators. Our results support premise 3 in the Introduction by showing that, after a relatively short

exposure to the metabolic activity of *M. reukaufii* genotypes, initial sucrose concentrations were obfuscated, with plant and yeast identity, and their interaction acquiring overwhelming explanatory power. These experimental results, however, may underestimate the actual impact of yeasts on individual variation in sugar concentration in the field. On one side, initial yeast cell densities in experimental units were lower than the densities commonly found in nectar of *H. foetidus* flowers exposed to pollinators (Herrera et al. 2008, 2009). And on the other, length of exposure to yeasts in this study was much shorter than natural lifetime of individual *H. foetidus* flowers (up to 20 d; Herrera et al. 2001). Since sucrose concentration of *H. foetidus* nectar declines as yeast cell density rises with flower age (Herrera et al. 2008), effects in the field could perhaps be larger than those reported here.

Floral nectar is a major pollinator reward that strongly influences plant-pollinator interactions, and nectar features have been mostly interpreted in the light of selection from pollinators (Nicolson et al. 2007, Abrahamczyk et al. 2017). More recently, however, the potential role of nectar microorganisms in nectar evolution has begun to be recognized, but our understanding of their actual role is still in an incipient stage (Canto and Herrera 2012, Parachnowitsch et al. 2019, Barberis et al. 2024). Results of this study have revealed an hitherto unrecognized role of specialized nectar yeasts in nectar trait evolution: by reshaping intrinsic individual differences among plants, *M. reukaufii* yeasts will reduce the opportunity for pollinator selection on *H. foetidus* nectar sugar concentration. Such reduction will hinder selection on the plants' intrinsic values of nectar sugar content, as their variation will have been "masked" by the transformative action of the yeast genotypes on nectar. Such reduction in opportunity for selection, however, will be in a sense compensated by new opportunities on an emergent axis. The variation among yeast genotypes in sugar transformative capacity, plus the strong plant x yeast genotype interaction effect on sugar concentration, will open opportunities for selection on

sugar concentration over the two-dimensional space defined by the combinations of plant x yeast genotypes (Appendix S1: Figure S1). In this proposed scenario, pollinator selection on nectar traits of plant x yeast combinations will supersede "naïve" pollinator selection on intrinsic nectar traits of individual plants, since the plant nectar phenotypes encountered by pollinators will largely be extended phenotypes of the yeast genotype(s) within the nectar. Nectar features could thus be considered as extended phenotypes of yeasts insofar as they are "candidate adaptations for the benefit of alleles responsible for variation in them" (Dawkins 2004), as discussed below.

Since initial conditions (sucrose concentration, yeast cell density) had a negligible effect on our experiment results, it seems safe to conclude that variation among experimental units in sucrose concentration after exposure to yeasts mainly arose from differences among yeast genotypes in growth rate and metabolic competence to exploit not just sucrose, but also other influential nectar constituents such as monosaccharides, proteins, amino acids and lipids which also occur in the nectar of *H. foetidus* but were not measured here (Herrera 1989, Vesprini et al. 1999). The concentration of these other nectar constituents probably varied among plants, and in our experiment their collective, unmeasured effects are subsumed under the "plant identity" factor. Yeast genotypes with metabolic profiles variably suited to the chemical profile of a given plant's nectar will probably grow and modify sucrose concentration at different rates (Herrera 2014). All else being equal, therefore, bumble bee discrimination for nectars with different sucrose concentrations (Cnaani et al. 2006, Whitney et al. 2008, Pozo et al. 2020) will translate into selection among plant-yeast combinations which have different sucrose concentrations (i.e., among different tiles in Appendix S1: Figure S1). As bumble bees simultaneously disperse yeast propagules and pollen grains, any positive selection on a particular plant-yeast combination arising from pollinator discrimination will automatically benefit both the plant and the yeast genotype(s) involved, contribute to consolidate their association, increase its frequency, and

perhaps eventually become "stabilized" within *H. foetidus* populations over the blooming season. In the long run, differential dispersal success of yeast genotypes associated with different plant individuals, and the likely stabilization of plant-yeast genotypic pairs postulated here, could eventually contribute to the genetic differentiation of *M. reukaufii* subpopulations found in flowers of *H. foetidus* individuals growing side by side, as well as the remarkable genetic and phenotypic diversity that characterize this yeast species (Herrera 2014, Herrera et al. 2014, Pozo et al. 2015, Dhami et al. 2018).

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Author contributions

C. M. H. conceptualized the study, performed field sampling, set up the experiment, analyzed the data and led writing. C. A. set up and conducted sugar analytical work. Both authors contributed to data curation, edited and revised manuscript drafts and approved the final version.

Conflict of interest statement

Authors declare no conflict of interest.

Data availability statement

Data and metadata are available at figshare (to be submitted)

ORCID

Carlos M. Herrera https://orcid.org/0000-0003-2452-3888

Conchita Alonso https://orcid.org/0000-0002-7418-3204

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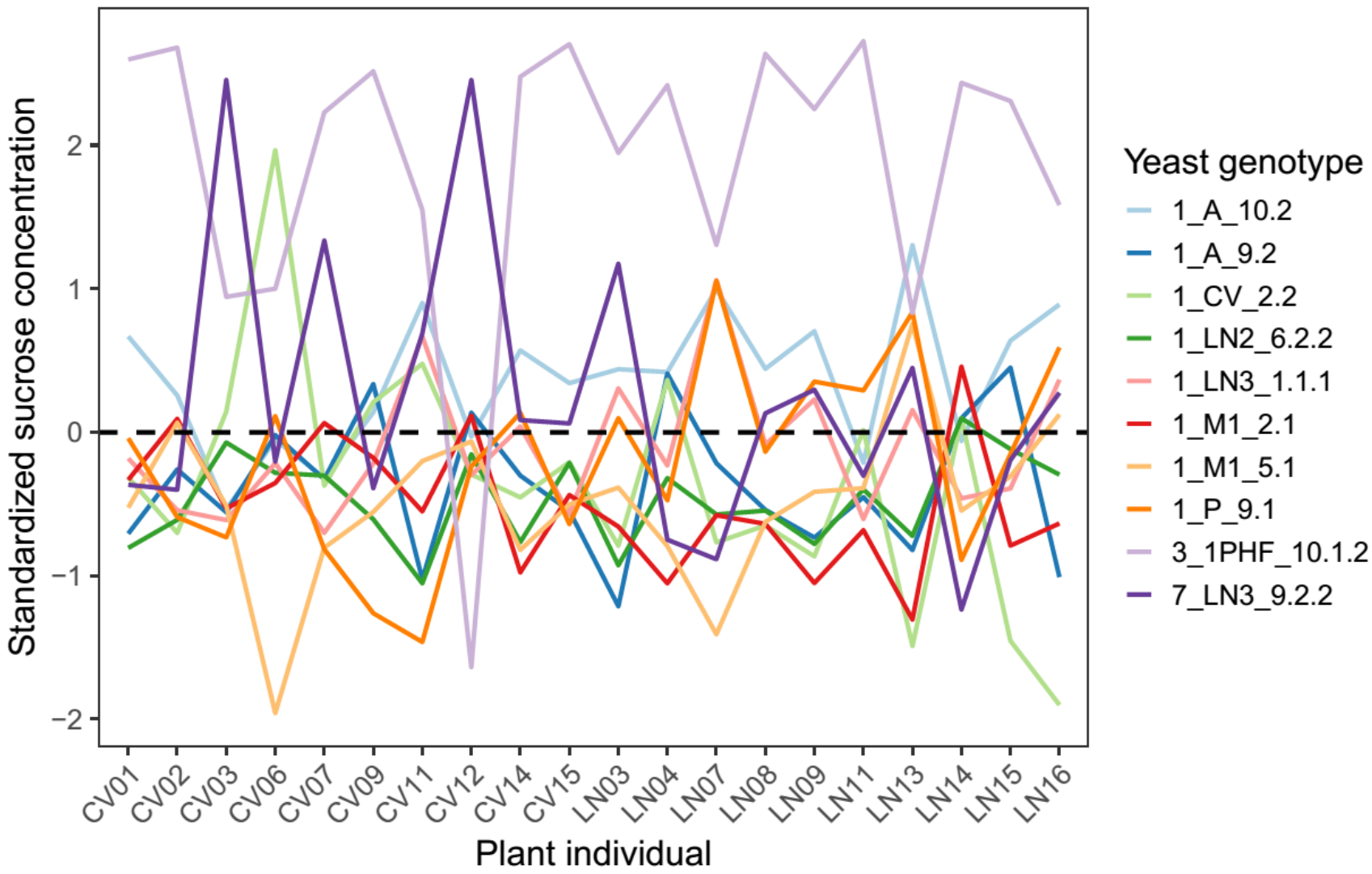
Table 1. Summary of statistical analyses testing for effects on nectar sucrose concentration after experimental exposure of nectar from 20 different plants to 10 yeast genotypes using a factorial design. Separate models were fitted to the data (*N* = 786 sugar concentration measurements) in which the predictors were initial yeast cell density and sucrose concentration, and plant and yeast genotype identities, respectively.

Predictors in model	Effect	Parameter estimate (SE)	Test statistic	*P*-value	Variance explained (%)
Initial yeast and nectar features:			Student's *t*		
	Cell density (CD)	-0.0117 (0.009)	1.35	0.18	2.1
	Sucrose concentration (SC)	0.289 (0.13)	2.26	0.024	9.1
	CD x SC	0.0001 (0.001)	0.84	0.39	0.084
Plant and yeast identity:			*F* value (df)		
	Plant (P)		31.6 (19, 586)	< 2E-16	32.5
	Yeast genotype (YG)		31.7 (9, 586)	< 2E-16	15.3
	YG x P		2.2 (171,586)	7.9E-12	20.3

Caption to the figure

Figure 1. Interaction plot depicting differences in sucrose concentration after experimental exposure of nectar from different plants to different yeast genotypes. To emphasize the contrasting effect of the different yeast genotypes on different individual plants, and to facilitate comparisons across plants, model-predicted sucrose concentrations for each plant x yeast genotype combination were scaled and centered separately for each plant. See Appendix S1: Figure S1, for the distribution of model-predicted values of sucrose concentration over the two-dimensional space defined by the 20 plants and 10 yeast genotypes tested in the experiment.

Figure 1


Standardized sucrose concentration
2
1
0
−1
−2
CV01
CV02
CV03
CV06
CV07
CV09
CV11
CV12
CV14
CV15
LN03
LN04
LN07
LN08
LN09
LN11
LN13
LN14
LN15
LN16
Plant individual
Yeast genotype
1_A_10.2
1_A_9.2
1_CV_2.2
1_LN2_6.2.2
1_LN3_1.1.1
1_M1_2.1
1_M1_5.1
1_P_9.1
3_1PHF_10.1.2
7_LN3_9.2.2