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De novo Synthesis of Benzenoid Compounds by the yeast *Hanseniaspora vineae* Increases Flavor Diversity of Wines

Running Title: Benzenoid compound synthesis by yeast increases flavor

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ABSTRACT

Benzyl alcohol and other benzenoid-derived metabolites of particular importance in plants confer floral and fruity flavors to wines. Amongst the volatile aroma components in *Vitis vinifera* grape varieties, benzyl alcohol is present in its free and glycosylated forms. These compounds are considered to originate from grapes only and not from fermentative processes. We have found increased levels of benzyl alcohol in red Tannat wine compared to grape juice, suggesting *de novo* formation of this metabolite during vinification.

In this work, we show that benzyl alcohol, benzaldehyde, *p*-hydroxybenzaldehyde and *p*-hydroxybenzyl alcohol are synthesized *de novo* in the absence of grape-derived precursors by *Hanseniaspora vineae*. Levels of benzyl alcohol produced by 11 different *H. vineae* strains were twenty to two hundred times higher than those measured in fermentations with *Saccharomyces cerevisiae* strains. These results show that *H. vineae* contributes to flavor diversity by increasing grape variety aroma concentration in a chemically defined medium. Feeding experiments with phenylalanine, tryptophan, tyrosine, p-aminobenzoic acid and ammonium in an artificial medium were tested to evaluate the effect of these compounds either as precursors or as potential pathway regulators for the formation of benzenoid-derived aromas. Genomic analysis shows that the phenylalanine ammonia-lyase (*PAL*) and tyrosine ammonia lyase (*TAL*) pathways, used by plants to generate benzyl alcohols from aromatic amino acids, are absent in *H. vineae* genome. Consequently, alternative pathways derived from chorismate with mandelate as an intermediate are discussed.

Keywords: benzyl alcohol, wine yeast fermentation, *Hanseniaspora vineae*, genome, *PAL/TAL* alternative biosynthetic pathway.

INTRODUCTION

The importance of volatile aryl alkyl alcohols in the flavor and grape character of some cultivars of *Vitis vinifera* is well reviewed.[1-3] The dominating aryl alkyl alcohols found in several grape varieties are the aromatic group of benzenoid/phenylpropanoid-related compounds (intermediates and end products) that significantly contribute to wine aroma during vinification or barrel aging.[1] Benzenoid/phenylpropanoid compounds, such as β-phenylethyl alcohol and benzyl alcohol can represent 10% to 51% of the total hydrolyzed volatile fraction of grapes such as Chardonnay,[4] Cabernet Sauvignon, Merlot,[5] Tannat[6] and Pinot Noir [7], contributing with flavors notes described as floral or fruity.[2, 6-8] Plant benzenoids also provide numerous specialized metabolites that participate in many key functions such as plant-plant communication, antimicrobial activity, phytohormones, vitamins, plant defense, etc.[9]

Some winemakers are rediscovering the value of using mixed cultures or spontaneous fermentation to increase yeast diversity, expecting to result in increased flavor complexity.[10-13] Understanding of their impact of non-*Saccharomyces* yeast strains on wine flavor richness is still incipient[14-16], although their account for more than 99% of the grape native flora. Furthermore, within non-*Saccharomyces* grape natural flora, the morphologic apiculate yeast of the genus *Hanseniaspora,* accounts for approximately 60% or more of this natural flora.[13]

We have recently demonstrated that during white Chardonnay wine vinification, application of *Hanseniaspora vineae* increased some aroma compounds such as β-phenylethyl acetate, compared to conventional fermentations [10, 17]. Moreover, during our studies of red wine fermentations with the typical grape cultivar Tannat of Uruguay, we have found higher levels of total benzyl alcohol in wine compared to grape juice,[6, 18] suggesting *de novo*

formation of this metabolite during vinification.

Although the complete metabolic pathways leading to the formation of volatile benzenoids is still not totally understood, it is known that benzyl alcohol is formed in plants within the phenylpropanoid synthesis by the *PAL* enzyme (phenylalanine ammonia-lyase).[9] This enzyme is the first of phenylpropanoid metabolism in plants, and catalyzes the conversion of phenylalanine to trans-cinnamic acid[19], which is subsequently converted into benzyl alcohol and other derived compounds. It has been found in some Basidiomycota and Ascomycota fungi.[20, 21] This enzyme is rarely found in yeast, and although it has been reported for the Basidiomycota yeast *Rhodotorula graminis*,[22] it has not been found in the subphylum Saccharomycotina. Only one study in a defined medium reported the formation of benzyl alcohol for some species of Saccharomycotina: *Kloeckera apiculata (Hanseniaspora uvarum*), *Candida stellata*, *Schizosaccharomyces* and *Zygosaccharomyces*.[23] Moreover, *p*-hydroxybenzoate has been shown to be an intermediate of ubiquinone Q6 synthesis in yeast[24, 25], although the formation pathway is not totally understand. With labeling experiments it was reported that *p*-hydroxybenzoate is synthesized mainly through the shikimate/chorismate pathway, and mutants (*aro1* and *aro2*) in this pathway are able to convert Tyr to *p*-hydroxybenzoate and efficiently compensate this situation (see Figure 1).[26] In this figure it is shown that *Saccharomyces* uses the chorismate biosynthesis pathway for the synthesis of Phe, Tyr, Trp and *p*-ABA.[27]

Benzyl alcohol (BAL) has been associated in wine varieties such as Cabernet Sauvignon,[5] to aroma descriptors such as chocolate, fig and tobacco, while benzaldehyde (BD) has been known to contribute to almond and dry fruit aroma descriptors. On the other hand, *p*-hydroxybenzyl compounds were described as fruity-sweet coconut, and woody or vanilla flavors.[28] Some of these descriptors have been also identified in Tannat wines before and

after malolactic fermentation.[29]

In the search for new non-*Saccharomyces* yeast strains with the capacity to produce aromatic compounds in grape musts with low nitrogen content, in the present work we show that *H. vineae* strains can synthesize benzylic and *p*-hydroxybenzyl alcohols *de novo* in higher concentrations than those found in grapes, thus contributing to an increase of yeast flavor after vinification. Phenolic amino acids and ammonium concentration changes in the artificial medium were also tested to evaluate the effect of these compounds as potential pathway regulators for the formation of these benzenoid compounds. As we have sequenced the genome of *H. vineae*,[30] putative pathways used by it to synthesize benzenoids are discussed in comparison with *S. cerevisiae* data and plants.

MATERIALS AND METHODS

Yeast strains

S. cerevisiae strains used were: Montrachet UCD 522 (University of California, Davis), referred to as M522 in this work; ALG 804 commercial strain (DSM, Denmark), EC1118 (Lallemand, Canada); and 881, 882 and KU1, selected Uruguayan wine strains that were already genetically characterized.[31] All these *S. cerevisiae* strains are used in the commercial production of wine. *H. vineae* strains were isolated from vineyards in Uruguay and the codes indicated in the figures are from our Enology Section's yeast culture collection. The 11 *H. vineae* isolates were identified by sequencing the variable D1/D2 region from 26S rDNA gene and differentiated as different strains with Tandem repeat tRNA PCR analysis.[32]

Fermentation conditions

Chemically defined synthetic grape fermentation medium (resembling the nutrient composition of grape juice but devoid of grape-derived secondary metabolites) was used

148 and referred to as CDG medium in this work. It was prepared as described previously,[33] but
149 modified as follows: the total nitrogen content was adjusted to a basic amount of yeast
150 assimilable nitrogen (YAN level, sum of amino acids and ammonium without proline) of
151 50 mgN/L for the experiments with diammonium phosphate (DAP) additions, 150 mgN/L
152 simulating an industrial grape juice and 100 mgN/L for studying phenolic amino acids and
153 *p*-aminobenozoic acid (*p*-ABA) effects. The latter YAN concentration was used because it
154 resulted in higher benzenoids formation. YAN values were indicated for each experiment,
155 and shown here are the following concentrations for each nitrogen component to obtain a
156 CDG medium with a YAN level of 100 mgN/L, expressed in mg/L: ammonium phosphate
157 (60.3), phenylalanine (18.1), tryptophan (12.1), tyrosine (2.4), leucine (36.2), arginine
158 (90.4), aspartate (42.2), glutamate (60.3), serine (48.2), threonine (42.2), lysine (30.1),
159 glutamine (24.1), isoleucine (24.1), valine (24.1), histidine (18.1), asparagine (18.1),
160 methionine (18.1), proline (60.3), alanine (12.1) and glycine (6)., This medium contains
161 0.2 mg/L of *p*-ABA, and the final pH of each medium was adjusted to 3.5 with HCl.
162 Equimolar concentrations of glucose and fructose were added to reach 200 g/L and the
163 mixed vitamins and salts were as described previously.[34] Ergosterol was added as the only
164 supplemented lipid at a final concentration of 10 mg/L. Inocula were prepared in the same
165 CDG medium with 100 mgN/L YAN by incubation for 12 h in a rotary shaker at 150 rpm
166 and 25 °C. Inoculum size was 1×10^5 cells/mL in the final medium for all strains as it was
167 defined to improve aroma production in this medium [35]. Fermentations were carried out in
168 125 mL of medium contained in 250 mL Erlenmeyer flasks, closed with cotton plugs to
169 simulate microaerobic conditions.[36] Fermentations were conducted in static batch
170 conditions at 20 °C in triplicate. Samples were taken once a day to measure cell growth in

171 an improved Neubauer chamber. Fermentation activity was measured as CO_2 weight loss
172 and expressed in grams per 100 mL. Samples for GC-MS analysis were taken one day after
173 fermentation was completed (except for experiments in Figure 2b as indicated), SO_2 was
174 added as 50 mg/L of sodium metabisulfite, filtered through 0.45 mm pore membranes and
175 kept at 4 °C until analyzes.

176 ***Feeding experiment effects on benzenoid compounds***

177 DAP, phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp) and *p*-ABA were chosen as the
178 YAN variables for the investigation of the effect of these amino acids and ammonium on
179 the production of BAL, benzaldehyde (BD), *p*-hydroxybenzyl alcohol (*p*-HBAL) and *p*-
180 hydroxybenzaldehyde (*p*-HBD). Experiments with different concentrations of each amino
181 acid were performed always using a 100 mgN/L YAN concentration, substituting the amino
182 acid concentration with DAP when the corresponding amino acid was not added, and
183 decreasing the concentration of DAP in the medium when amino acids were added.
184 Analysis of the effect of ammonium on benzenoid compound production was performed at
185 different YAN concentrations of 50, 75 and 250 mgN/L by supplementation with DAP,
186 using the same feeding levels as our previous work with *S. cerevisiae*.[33]

187 ***GC-FID and GC-MS analysis***

188 ***Extraction of aroma compounds***. It was performed using adsorption and separate elution
189 from an Isolute (IST Ltd, Mid Glamorgan, UK) ENV1 cartridge packed with 1 g of a highly
190 cross-linked styrene-divinyl benzene (SDVB) polymer. Treatment of samples and GC-MS
191 analysis were performed as described previously[6] in a Shimadzu-QP 2010 ULTRA (Tokyo,
192 Japan) mass spectrometer equipped with a Stabilwax (30 m × 0.25 mm i.d., 0.25 µm film
193 thickness, Restek) capillary column.

194 ***Identification and quantification***. GC-FID and GC-MS instrumental procedures using an
195 internal standard (1-heptanol) were applied for quantitative purposes, as described
196 previously.[6] The components of wine aromas were identified by comparison of their linear
197 retention indices, with pure standards for BAL, BD, *p*-HBAL alcohol and *p*-HBD (Aldrich,
198 Milwaukee, WI). Comparison of mass spectral fragmentation patterns with those stored on
199 databases was also performed.

200 ***Hanseniaspora vineae genome and gene annotation***

201 *H. vineae* strains were isolated during fermentation of wine made from Tannat, the
202 traditional red grape of Uruguay.[18] Genomic DNA from strain T02/19AF was sequenced on
203 an Illumina Genome Analyzer IIx platform and the processed reads were then assembled
204 using MaSuRCA,[37] as was reported recently by us.[30] The putative open reading frames
205 predicted with Augustus[38] were annotated through blastp against *S. cerevisiae* S288C
206 proteins (see supplementary material). PAL/TAL genes and chorismate pyruvate lyase were
207 searched using the following Uniprot sequence: P11544 (PAL/TAL, of *Rhodotorula*
208 *gracilis*) and P26602 (chorismate pyruvate lyase, of *Escherichia coli*). Protein domains
209 were annotated using the pfam database (http://pfam.xfam.org/) (see supplemental
210 material). To establish orthologous clusters between *S. cerevisiae* S288C and *H. vineae*, the
211 predicted proteins were analyzed through OrthoMCL web server (see supplementary
212 material)[39]. This whole-genome shotgun project has been deposited at
213 DDBJ/EMBL/GenBank under the accession number JFAV00000000.

214 The presence and absence of genes was discussed according to the annotation of the
215 genomic sequences of *H. vineae* strain. Table 1 was built with *H. vineae* genome compared
216 with the industrial wine yeast *S. cerevisiae* EC 1118 genome that was the strain used for the
217 feeding experiments with amino acids and *p*-ABA shown in Figures 5 and 6.

Statistical analysis

ANOVA analysis of BAL in wine produced using 16 yeast strains in the CDG fermentation medium was performed. ANOVA of the effect of DAP addition on benzenoid compounds at three YAN levels (50, 75 and 250 mgN/L) for *H. vineae* was also analyzed. The benzenoid compound concentrations were also evaluated for the effects of Phe, Tyr, Trp and *p*-ABA in the CDG medium. STATISTICA 7.0 software was used for all the ANOVA analyses. Differences in mean benzenoid compound concentrations were evaluated by the least significant differences test.

RESULTS AND DISCUSSION

We selected 11 different *H. vineae* strains from our native yeast collection to compare within the species the capacity for formation of BAL in the synthetic medium. The production of BAL by wine yeasts was measured at the end of fermentation in CDG medium containing 150 mgN/L YAN (a usual YAN level found in industrial grape juice) (Figure 2). Five native and commercial wine *S. cerevisiae* strains were compared to 11 *H. vineae* strains including Hv025 previously applied by us for winemaking.[17] It can be seen that the accumulation of BAL by *S. cerevisiae* strains is very limited in the CDG medium compared to the tested *H. vineae* strains. Figure 2 shows that the production of this alcohol within the species *H. vineae* varies in concentration from 87 up to 620 µg/L in these conditions. The highest producer, strain Hv12196, produced a concentration level above the average sum of free and bound forms found in Tannat grape juice (385 µg/L) as previously described.[40] When relating the production of BAL to fermentation rates (estimated as average rate between day 3 and 13, gCO_2/100 mL.day for each strain of *H. vineae*, Fig 2) and total biomass (BAL/cell, Figure 3a), it was confirmed that there was no correlation with these parameters (R^2=0,51 and R^2=0,61 respectively). Figure 3b shows the

242 formation and accumulation of BAL and its acetate ester during fermentation. Interestingly,
243 acetylation of this alcohol shows a very low formation rate (around 2% of the alcohol was
244 acetylated) when compared to the acetylation of β-phenylethyl alcohol that was detected
245 previously up to 50% in *H. vineae* strains, resulting in significantly higher concentrations of
246 β-phenylethyl acetate.[41, 42]

247 ***Effect of ammonium on BAL and p-BAL production***

248 Feeding experiments with changes in ammonium concentration in the artificial medium
249 were designed to investigate the effect of this compound as a potential pathway regulator
250 for the formation of BAL, BD, *p*-HBAL and *p*-HBD for the main producer strain 12/196.
251 Figure 4 shows the effect of ammonium; where a consistent negative correlation is
252 observed in the formation of these four compounds with DAP levels (BAL R^2=0,85; p-
253 HBAL R^2=0,95; BD R^2=0,84 and p-HBD R^2=0,87). A significant decrease of BAL and *p*-
254 HBAL formation is observed with a YAN level of 250 mgN/L (78 and 22 µg/L,
255 respectively). Experiments with the same YAN (250 mgN/L), but with the amino acid
256 mixture instead of DAP addition, result in a weaker decrease of BAL formation (170 µg/L,
257 data not shown). The similar behavior of both aldehydes, but at very low concentrations,
258 suggested that they could be intermediates of alcohol formation. This behavior in both
259 alcohols suggests an inhibitory effect or metabolic re-orientation of inorganic nitrogen at
260 some step in the biosynthetic pathway, as was shown for the formation of other higher
261 alcohols in *S. cerevisiae*.[33] However, BAL does not show a similar pattern at very low YAN
262 levels between 50 and 75 mgN/L, where an increase of production of the main higher
263 alcohols was shown.[33] BAL formation increased with the lowest YAN level up to
264 1055 µg/L (Figure 4) compared to 620 µg/L at 150 mgN/L YAN (Figure 2). As was stated in
265 the introduction, to the best of our knowledge, only one reference was found reporting the

production of BAL in a synthetic medium of up to 464 µg/L with the yeast *Schizosaccharomyces pombe*.[23] In agreement with these results, although concentrations produced by *S. cerevisiae* yeasts were very low, the significant decrease of BAL formation by DAP and anaerobic conditions was reported by us also for this species.[36] Avoiding or delaying nitrogen supplementation during winemaking could be an interesting strategy to increase benzenoid synthesis for the final wine. Implications of DAP addition on decreasing phenolic aroma compounds formation during winemaking are further discussed by us (Martin et al.) [43]

Effect of Phe, Tyr, Trp and p-ABA on BAL, p-HBAL, BD and p-HBD production

Feeding experiments were designed to investigate the effect of these compounds either as potential precursors and/or pathway regulators for the formation of BAL and BD (considering phenylpyruvate as a probable precursor), and *p*-HBAL and *p*-HBD (considering *p*-hydroxyphenyl pyruvate as the other probable precursor). In Figure 5, the effect of omitting and doubling the concentration of each of the three aromatic amino acids in the medium, keeping YAN constant at 100 mgN/L, is shown. As it was expected for *H. vineae*, BAL was produced, yielding more than 30-fold higher concentration than the industrial strain of *S. cerevisiae* EC 1118. It could be observed for *H. vineae* that Phe and *p*-ABA addition significantly increased the production of BAL and Tyr, in the contrary decreased it. The significant increase of BAL formation in *H. vineae* with Trp addition at low concentration may be explained if *de novo* synthesis of this alcohol occurs from sugars. It is known for some *S. cerevisiae* strains that Trp is a stimulator of the initial steps of the chorismate pathway unlike Phe and Tyr which are inhibitors[27], effects that are also in agreement with our results of Figure 5 for *S. cerevisiae* (EC1118 strain). This fact, and that Trp could not be a catabolic precursor of BAL, may support the hypothesis of *de novo*

290 synthesis of these compounds through an increase of chorismate availability in the pathway
291 (Figure 1 and 7). However, further research expanding the dose concentrations will be
292 needed with *H. vineae*, as Trp double-concentration decreased accumulation of BAL,
293 showing a bimodal behavior in Figure 5. The increase of *p*-ABA affects both species
294 equally, increasing BAL formation. It was recently reported for *S. cerevisiae* that changes
295 in *p*-ABA synthase gene (*ABZ1)* expression have a significant effect on the synthesis of
296 many flavor aroma compounds regulated by nitrogen, such as β-phenylethyl alcohol.[44] Our
297 results show a similar behavior of BAL and p-HBAL (and β-phenylethyl alcohol, data not
298 shown) upon *p*-ABA addition for *H. vineae*. However, an opposite behavior is observed
299 between the species upon increase of Phe. The significant increase of BAL with Phe double
300 concentration in *H. vineae* up to 1351 µg/L supports the hypothesis that Phe catabolism
301 capacity is increasing phenylpyruvate into the chorismate pathway. .
302 Figure 6, shows that production of *p*-HBAL by *H. vineae* is clearly another significant
303 difference with *S. cerevisiae*, yielding productions of two orders of magnitude higher, since
304 this compound in *Saccharomyces* final wines, was under a quantifiable value.
305 ***Benzyl and p-hydroxybenzyl alcohol formation pathways in Hanseniaspora vineae***
306 Three alternative routes to the *PAL/TAL* pathways were previously proposed to produce *p*-
307 hydroxybenzoate in *S. cerevisiae*: the direct formation from chorismate as in some bacteria
308 with a chorismate pyruvate-lyase,[45] a non-oxidative pathway proposed for plants with a
309 retro-aldol reaction from *p*-hydroxycoumarate and the peroxisome β-oxidative pathway
310 with acetyl CoA formation.[46] No evident gene homologies were found by us for the first
311 alternative in *H. vineae*. Furthermore, the second and third proposed alternatives were
312 excluded by the fact that both involve cinnamic acids or derived compounds such as
313 vinylphenol and vinylguaiacol that are unable to be synthesized by *S. cerevisiae* and *H.*

vineae in our CDG medium (data not shown). This observation strongly supports that these two pathways are not active in our experimental conditions. In addition, the key enzymatic step proposed by some authors for the conversion of *p*-hydroxyphenyl lactate to *p*-coumarate[24, 46] was never demonstrated for yeast.

The phenylpyruvate and p-hydroxyphenyl pyruvate pathways to benzenoids

Table 1 shows the presence and absence of aryl alkyl alcohol metabolism-related genes in *H. vineae* compared to *S. cerevisiae*. In Figure 7 we also show the proposed pathways for the formation of BAL and *p*-HBAL from phenylpyruvate and *p*-hydroxyphenyl pyruvate, respectively.

The synthesis of benzenoids in plants through phenylpyruvate as an alternative to the *PAL* pathway has been suggested within multiple putative pathways,[19] but has not yet been biochemically proved.[9] Furthermore, this pathway through phenylpyruvate was also proposed for the fungi *Bjerkandera adusta*;[47] however, it was also not proved, since this fungi also has the ability to form trans-cinnamic acid derivatives through *PAL*, as was stated for many basidiomycete and some ascomycete fungi.[20] In this report,[47] labeling studies from both Phe and cinnamic acid showed that Phe produced a higher percentage of labeled BAL in *B. adusta,* suggesting that Phe may be using two parallel pathways, through PAL and through phenylpyruvate, while the labeled cinnamic acid is using only the β-oxidative and/or the non-oxidative pathways (see Figure 7). *PAL* being absent in *H. vineae*, suggests that benzenoids are necessarily dependent on *de novo* synthesis from chorismate. Genomic analysis of *H. vineae* indicates the existence of all the genes that encode for enzymes of the chorismate pathway, and of *ARO7* (chorismate mutase) and *PHA2*/*TYR1* genes (see Figure 7), proving that this yeast could synthesize phenylpyruvate and *p*-hydroxyphenyl pyruvate through prephenate as in *S. cerevisiae*.

The mandelate pathway to benzenoids in yeast

Based on these studies and previous reports, we propose here the mandelate pathway as a non-cinnamic acid formation route to benzenoids, and as an alternative pathway for yeasts and other organisms that lack *PAL*. Although it was proposed for fungi with some unclear interpretations as discussed above,[47] the functionality of the mandelate pathway was just recently demonstrated in an engineered bacteria.[48] We proposed for *H. vineae* that *ARO10, SCS7* and *DLD1/2* genes may participate in this pathway. These genes are known to produce proteins with a wide functional capacity including reactions with mandelate and phenylpyruvate or phenyllactate[49, 50] Also, as ARO10 and DLD1 proteins are very divergent between *H. vineae* and *S. cerevisiae* (see Table 1), this may explain the *H. vineae'* s capacity for the synthesis of benzenoid compounds. The results presented here, the proven function of 4-hydroxybenzoate as an intermediate of ubiquinone, and the inability of both yeast species to synthesize cinnamic acids, are in agreement with the proposed pathway in Figure 7. Gene sequence divergences of these steps and the fact that these enzymes have also been shown to display activity on either phenylpyruvate or *p*-hydroxyphenyl pyruvate derived compounds[51, 52] clearly suggest a similar pathway for the formation of both alcohols. Following from the mandelate pathway yielding benzoate or *p*-hydroxybenzoate (or the corresponding aldehydes), BAL and *p*-HBAL can be formed by aryl alcohol dehydrogenases (Aad) or alcohol dehydrogenases (Adh).[53] The mandelate, β-oxidative and non-oxidative pathways mainly shown in plants and their relation with aromatic amino acids and the four benzenoids analyzed in this work are shown in figure 7. Further studies at biochemical level will be necessary to prove some of the steps that are indicated here for yeast.

In summary, *H. vineae* production of BAL and *p*-HBAL during wine fermentation was one

362 to two orders of magnitude higher than *S. cerevisiae* under the same fermentation
363 conditions in a chemically defined medium.
364 Phe/Trp/*p*-ABA and ammonium/Tyr concentration changes in the synthetic grape medium
365 composition showed an increase and a decrease, respectively, with the formation of BAL.
366 Modulation of these YAN nutrients could decrease or increase up to 17 times the total BAL
367 produced by *H. vineae*. Doubling Phe concentration in the medium significantly increased
368 BAL formation in *H. vineae* (up to 1351 µg/L), the opposite behavior compared to *S.*
369 *cerevisiae*. We propose that the formation of BAL in *H. vineae* could follow two alternative
370 origins, from sugars through chorismate and from active Phe catabolism, both routes with
371 phenylpyruvate as an intermediate and the subsequent formation of mandelate through
372 *ARO10* decarboxylase. Protein blast searches support these putative mechanisms that may
373 allow this species to synthesize these alcohols. The absence of the *PAL* and *TAL* routes and
374 the high BAL production by *H. vineae* makes it an ideal eukaryotic model to better
375 understand the synthesis of benzenoids, as compared to plants and fungi where coexistence
376 with the *PAL/TAL* pathways makes their metabolic analysis more complex to perform.
377
378 **ABBREVIATIONS USED**
379
380 BAL Benzyl alcohol ; BD benzaldehyde ; *p*-HBD *p*-hydroxybenzaldehyde ; *p*-HBAL *p*-
381 hydroxybenzyl alcohol; CDG chemically defined grape medium; YAN yeast assimilable
382 nitrogen; PHE phenylalanine; TYR tyrosine; TRP tryptophan; *p*-ABA *p*-aminobenozoic
383 acid; DAP Diammonium phosphate.
384 *ARO* genes requiring aromatic amino acid; *ARO10* phenylpyruvate decarboxylase; *ALD*
385 aldehyde dehydrogenase; *ABZ p*-ABA synthesis; *ADH* alcohol dehydrogenase; *ATF* alcohol
386 acetyl transferase; *BAT* branched-chain amino acid transaminase; *DLD* D-lactate
387 dehydrogenase; *GRE2* NADPH-dependent methylglyoxal reductase; *HOM2* aspartic beta
388 semi-aldehyde dehydrogenase; *OYE2* NADPH oxidoreductase; *PDC* pyruvate

389 decarboxylase; *PHA2* prephenate dehydratase; *SCS7* sphingolipid alpha-hydroxylase; *TYR*
390 prephenate dehydrogenase; *TRP* tryptophan synthesis; *SFA1* bifunctional alcohol
391 dehydrogenase and formaldehyde dehydrogenase.
392 In Figure 7: Phenylacetaldehyde (PheALD), *p*-hydroxyphenylacetaldehyde (*p*-HPheALD),
393 phenylacetic acid (PheAA), *p*-hydroxyphenylacetic acid (*p*-HPheAA), mandelic acid
394 (ManA), *p*-hydroxymandelic acid (*p*-HManA)
395

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FIGURE CAPTIONS

Figure 1

Shikimic acid and chorismate pathway for the synthesis of aromatic amino acids and *p*-aminobenozoic acid (*p*-ABA). The main intermediates are indicated in red: phenlypyruvate, *p*-hydroxyphenyl pyruvate and anthranilate. Green dotted lines indicate phenylalanine ammonia-lyase (*PAL*) and tyrosine ammonia-lyase (*TAL*) pathways that were proved in plants and some filamentous fungi, where the key intermediates to benzenoids are cinnamic acids. We confirmed here that these enzymes are not present in *Hanseniaspora vineae, Saccharomyces cerevisiae* and other Saccharomycotina yeasts. The great production of benzyl-derived compounds by *H. vineae* through an alternative pathway to *PAL* or *TAL* will contribute to understanding the steps that are shown with interrogation symbols on this scheme.

Figure 2

Benzyl alcohol (BAL) production by *Hanseniaspora vineae* strains compared to *Saccharomyces cerevisiae* wine yeasts (ALG804, KU1, Sc882, Sc881and M522,). Fermentations were performed in the model artificial grape medium with a yeast assimilable nitrogen (YAN) of 150 mgN/L at 20 °C, and samples were analyzed after 14 days of fermentation. Results showed significant difference between species ($P \leq 0.001$) according to an LSD test of ANOVA calculated for each strain. Error bars indicate SD of the mean value. Fermentation kinetics of the three most diverse (Hv0225, Hv12196 and Hv12213) and the two sequenced strains of *H. vineae* (Hv0205 and Hv0219) are shown as CO_2 weight lost, compared to M522 presented in dotted lines.

Figure 3

a. *Hanseniaspora vineae* strains and *Saccharomyces cerevisiae* M522 fermented in

chemically defined grape (CDG) medium with 150 mgN/L yeast assimilable nitrogen (YAN) at 20 °C; GC analysis was used to determine benzyl alcohol (BAL) production per cell. Error bars indicate SD of the mean value. **b.** Growth kinetics (dotted line) of *H. vineae* 02/25 under the same fermentation conditions. Benzyl acetate and BAL formation during fermentation are shown in bars at the end of the exponential phase (day 4) and at the end of the stationary phase (day 10). Error bars indicate SD of the mean value.

Figure 4

Benzyl alcohol (BAL), benzaldehyde (BD), *p*-hydroxybenzyl alcohol (*p*-HBAL) and *p*-hydroxybenzaldehyde *(p*-HBD) formation by *Hanseniaspora vineae* 12/196 in the chemically defined grape (CDG) medium with three yeast assimilable nitrogen (YAN) levels where 75 and 250 mgN/L levels were reached with diammonium phosphate (DAP) addition. Fermentations were carried out at 20 °C; data expressed in µg/L. Letters at each data point indicate the level of significant difference ($P \leq 0.001$) according to an LSD test of ANOVA calculated for each treatment. Error bars indicate SD of the mean value.

Figure 5

Benzyl alcohol formation by *Hanseniaspora vineae* 12/196 (left side) and by *Saccharomyces cerevisiae* EC1118 in the chemically defined grape (CDG) medium. Fermentations were performed at constant yeast assimilable nitrogen (YAN) level (100 mgN/L) but with omitted (0), normal juice concentration (1) or double-concentration (**2**) of the three amino acids phenylalanine (PHE), tyrosine (TYR) and tryptophan (TRP). Diammonium phosphate (DAP) was used as the substitute of each of these amino acids according to the treatment, to maintain 100 mg N/L. For *p*-aminobenozoic acid (*p*-ABA, 0.2 mg/L) experiments only with omitted and normal juice concentration were used. . Fermentation was carried out at 20 °C; data expressed in µg/L. Letters at each data point

indicate the level of significant difference ($P \leq 0.01$) according to an LSD test of ANOVA calculated for each treatment. Error bars indicate SD of the mean value.

Figure 6

p-hydroxybenzyl alcohol (*p*-HBAL), benzaldehyde (BD) and *p*-hydroxybenzaldehyde (*p*-HBD) formation by *Hanseniaspora vineae* Hv12/196 and by *Saccharomyces cerevisiae* EC1118 in the chemically defined grape (CDG) medium. Treatments with the same yeast assimilable nitrogen (YAN) level of 100 mgN/L in the omitted (0) and normal juice concentration (1) of phenylalanine (PHE, 18.1 mg/L), tyrosine (TYR, 2.4 mg/L) and tryptophan (TRP, 12.1 mg/L) Fermentations were carried out at 20 °C; data expressed in µg/L. Letters at data points indicate the level of significant difference ($P \leq 0.01$) according to an LSD test of ANOVA calculated for each treatment. Error bars indicate SD of the mean value. NQ: not quantifiable (below limit of quantification).

Figure 7

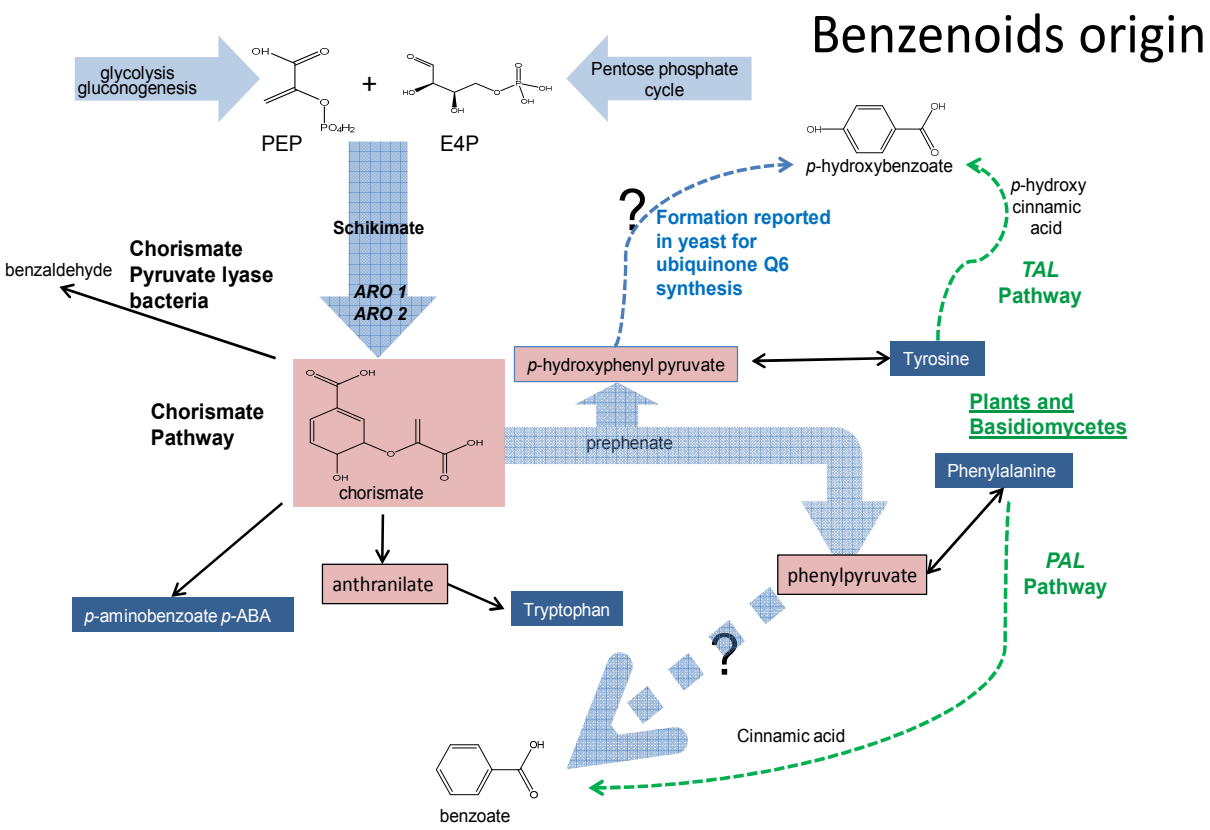
The proposed pathways for the formation of benzyl alcohol (BAL), benzaldehyde (BD), *p*-hydroxybenzyl alcohol (*p*-HBAL) and *p*-hydroxybenzaldehyde (*p*-HBD) in *Hanseniaspora vineae* through the chorismate pathway, with phenlypyruvate and *p*-hydroxyphenyl pyruvate as intermediates, are presented. In yellow, the presence of genes that codify for enzymes that catalyze different steps of the pathways were confirmed by genomic analysis of *H. vineae* compared with *Saccharomyces cerevisiae* EC 1118 (Table 1) and with plants. Compounds in red squares were determined by GC-MS in this work.

Table 1. Comparison of aryl alkyl metabolism-related genes of *H. vineae* and *S. cerevisiae* EC 1118 genomes.

class of compound	enzymatic activity	genes identified
higher alcohol	aromatic amino acid transferase	*ARO8* (59.84), *ARO9* (42.70)
	branched chain amino acid transferase	*BAT1* (78.84), ~~*BAT2*~~
	decarboxylase	*ARO10* (34.1), *PDC1* (80.46), ~~*PDC5, PDC6, THI3*~~
	alcohol dehydrogenase	~~*ADH1,*~~ *ADH2* (78.74), *ADH3* (74.80), ~~*ADH4, ADH5*~~, *ADH6* (44,74), ~~*ADH7*~~, *SFA1 (68.16), GRE2 (50.73)* ~~*YPR1*~~, ~~*PAD1*~~, ~~*SPE1*~~, *OYE2* (58.06), *HOM2* (78.24)
	aryl alcohol dehydrogenase	~~*AAD3, AAD4, AAD6, AAD10, AAD14, AAD15, AAD16*~~
	Regulation	*ARO80* (34.80), ~~*GAT2, GLN3, GZF3, DAL80*~~
acetate esters	alcohol acetyl transferases	~~*ATF1*~~, *ATF2 (26.58),*
volatile organic acids	aldehyde dehydrogenase	*ALD2* (44.01), ~~*ALD3, ALD4*~~, *ALD5 (53.45), ALD6* (55.07)
***p*-ABA synthesis**	synthesis of *p*-ABA from chorismate	*ABZ1* (40.59), *ABZ2* (35.52)
aromatic amino acid synthesis	synthesis of chorismate, phenylalanine tryptophan and tyrosine	*ARO1* (66.79), *ARO2* (80.59), *ARO3* (77.03), *ARO4* (83.51), *TRP2* (70.84), *TRP3* (69.14), *ARO7* (67.97), *PHA2* (41.99), TYR1 (62.37)
benzyl alcohol/ benzaldehyde synthesis	Mandelate pathway	*ARO10, PDC1, SCS7 (66.5), ALD6?, ALD2?, DLD1(53), DLD2(70),* ~~*DLD3*~~

Genes absent (crossed letters) or present are indicated; percentages in parentheses indicate amino acid sequence identity between both species. *ARO* genes requiring aromatic amino acid; *ARO10* phenylpyruvate decarboxylase; *ALD* aldehyde dehydrogenase; *ABZ p*-ABA synthesis; *ADH* alcohol dehydrogenase; *ATF* alcohol acetyl transferase; *BAT* branched-chain amino acid transaminase; *DLD* D-lactate dehydrogenase; *GRE2* NADPH-dependent methylglyoxal reductase; *HOM2* aspartic beta semi-aldehyde dehydrogenase; *OYE2* NADPH oxidoreductase; *PDC* pyruvate decarboxylase; *PHA2* prephenate dehydratase; *SCS7* sphingolipid alpha-hydroxylase; *TYR* prephenate dehydrogenase; *TRP* tryptophan synthesis; *SFA1* bifunctional alcohol dehydrogenase and formaldehyde dehydrogenase

Figure 1


Benzenoids origin
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gluconogenesis
PEP
+
E4P
Pentose phosphate
cycle
Schikimate
ARO 1
ARO 2
Chorismate
Pyruvate lyase
bacteria
benzaldehyde
Chorismate
Pathway
chorismate
anthranilate
Tryptophan
p-aminobenzoate p-ABA
p-hydroxybenzoate
?
Formation reported
in yeast for
ubiquinone Q6
synthesis
p-hydroxy
cinnamic
acid
TAL
Pathway
p-hydroxyphenyl pyruvate
Tyrosine
prephenate
Plants and
Basidiomycetes
Phenylalanine
phenylpyruvate
PAL
Pathway
?
Cinnamic acid
benzoate

Figure 2

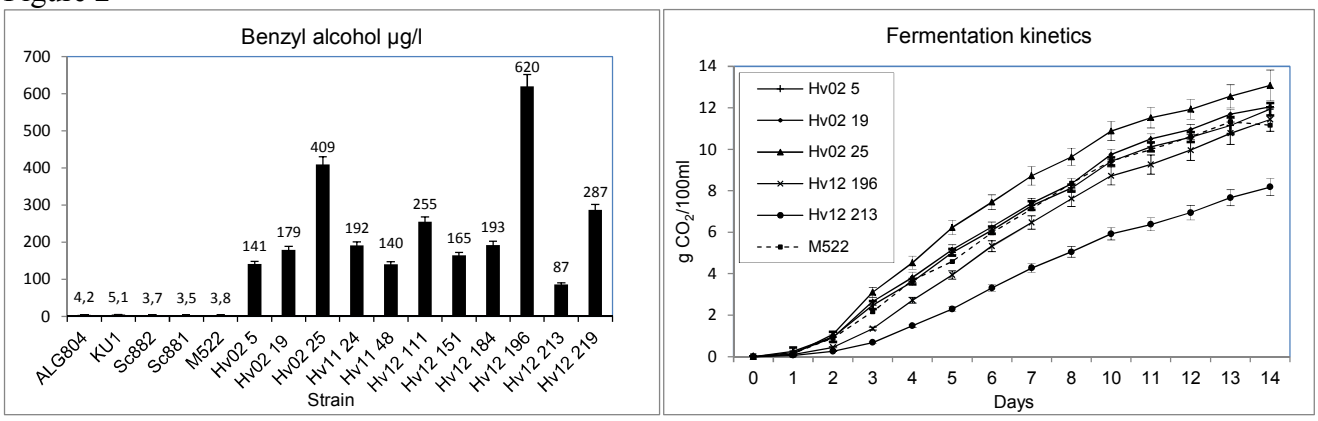


Figure 3

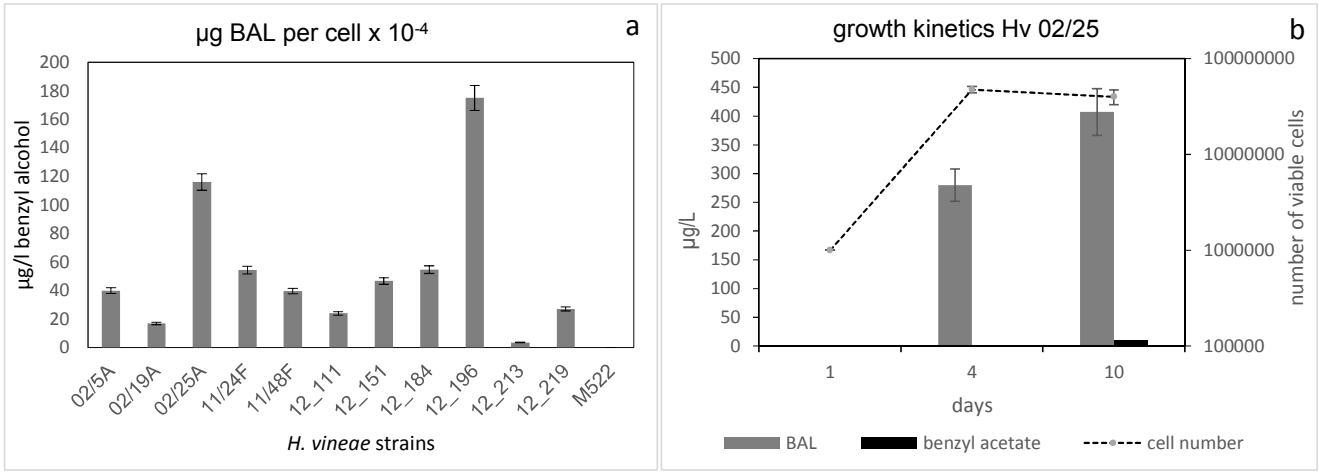


Figure 4

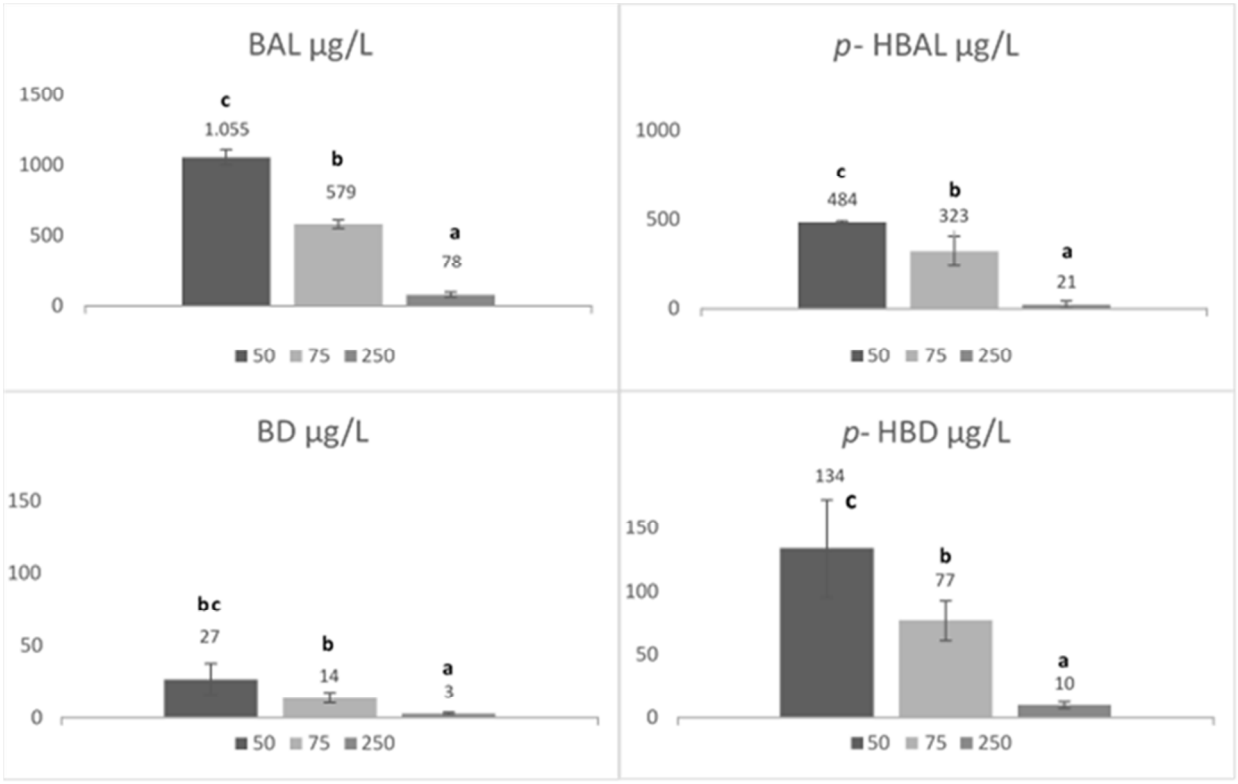
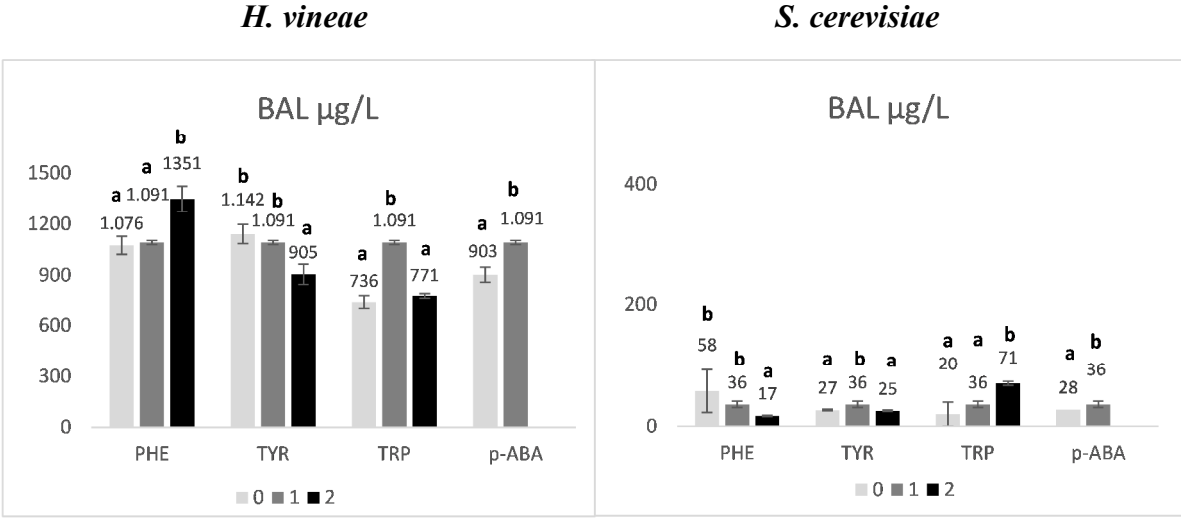

BAL µg/L
1500
1000
500
0
c
1.055
b
579
a
78
50 75 250
p- HBAL µg/L
1000
500
0
c
484
b
323
a
21
50 75 250
BD µg/L
150
100
50
0
bc
27
b
14
a
3
50 75 250
p- HBD µg/L
134
c
150
100
50
0
b
77
a
10
50 75 250

Figure 5


H. vineae
S. cerevisiae
BAL µg/L
1500
1200
900
600
300
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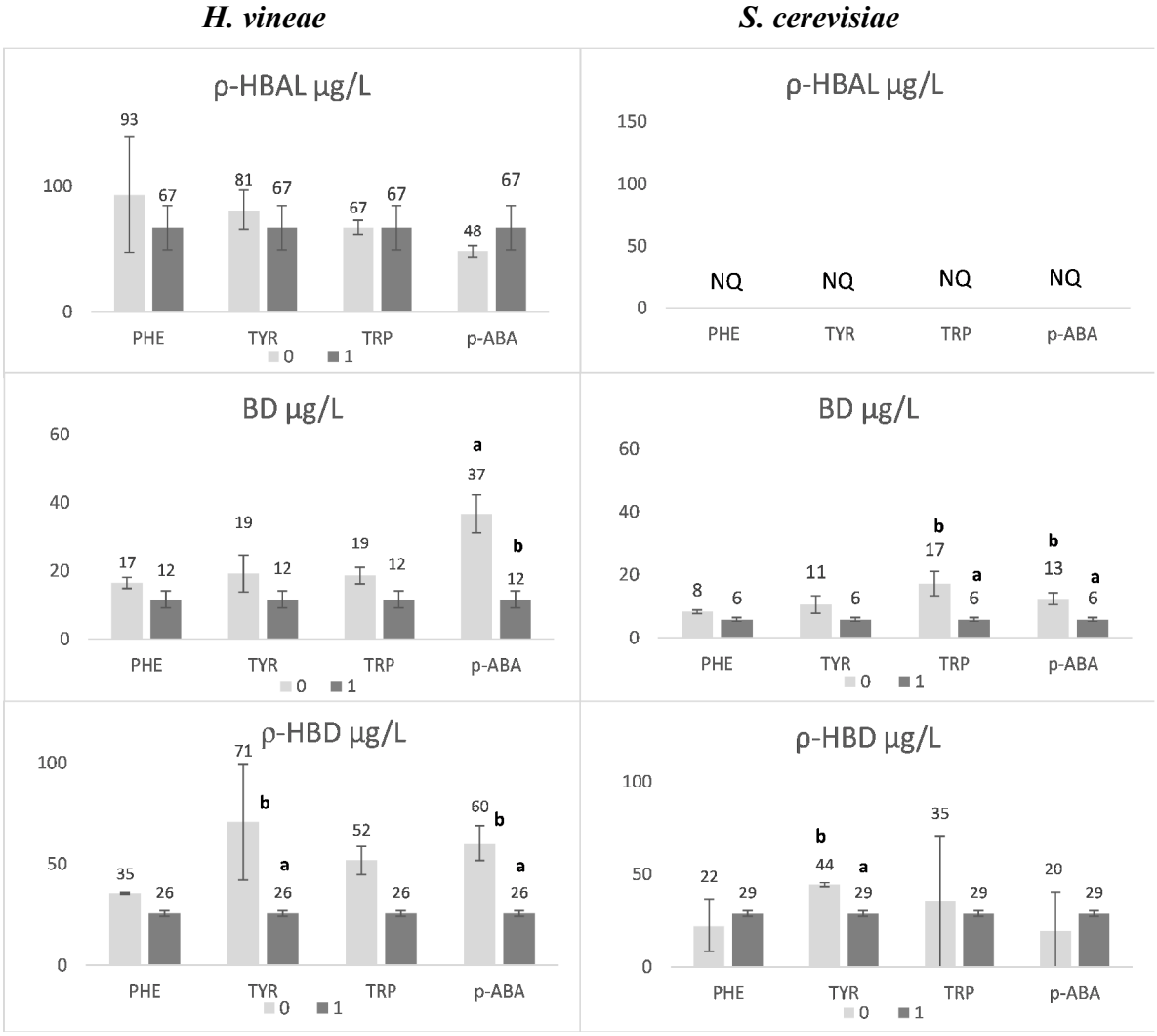
a 1.076
a 1.091
b 1351
b 1.142
b 1.091
a 905
a 736
b 1.091
a 771
a 903
b 1.091
PHE
TYR
TRP
p-ABA
0 1 2
BAL µg/L
400
200
0
b 58
b 36
a 17
a 27
b 36
a 25
a 20
a 36
b 71
a 28
b 36
PHE
TYR
TRP
p-ABA
0 1 2

Figure 6


H. vineae
S. cerevisiae
ρ-HBAL µg/L
93
67
81
67
67
67
48
67
100
0
PHE
TYR
TRP
p-ABA
0
1
ρ-HBAL µg/L
150
100
50
0
NQ
NQ
NQ
NQ
PHE
TYR
TRP
p-ABA
BD µg/L
60
40
20
0
17
12
19
12
19
12
a
37
b
12
PHE
TYR
TRP
p-ABA
0
1
BD µg/L
60
40
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0
8
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11
6
b
17
a
6
b
13
a
6
PHE
TYR
TRP
p-ABA
0
1
ρ-HBD µg/L
100
50
0
35
26
71
b
a
26
52
26
60
b
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26
PHE
TYR
TRP
p-ABA
0
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ρ-HBD µg/L
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0
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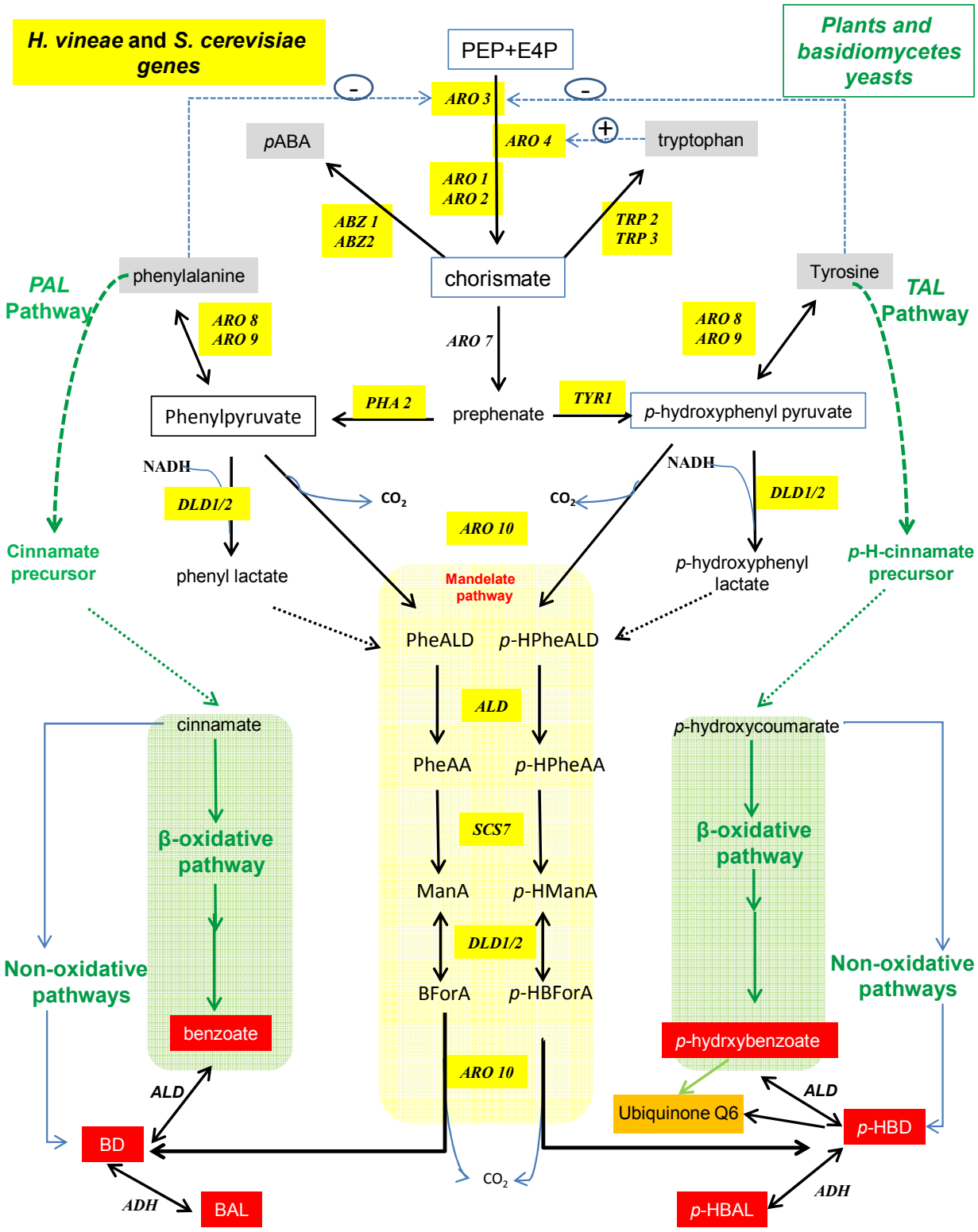
PHE
TYR
TRP
p-ABA
0
1

Figure 7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Plants and basidiomycetes yeasts
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ARO 4
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ARO 2
pABA
tryptophan
ABZ 1
ABZ2
TRP 2
TRP 3
chorismate
PAL Pathway
phenylalanine
Tyrosine
TAL Pathway
ARO 8
ARO 9
ARO 7
Phenylpyruvate
PHA 2
prephenate
TYR1
p-hydroxyphenyl pyruvate
NADH
DLD1/2
CO2
ARO 10
Cinnamate precursor
phenyl lactate
p-hydroxyphenyl lactate
p-H-cinnamate precursor
Mandelate pathway
PheALD
p-HPheALD
ALD
cinnamate
p-hydroxycoumarate
PheAA
p-HPheAA
SCS7
β-oxidative pathway
ManA
p-HManA
DLD1/2
BForA
p-HBForA
Non-oxidative pathways
benzoate
p-hydrxybenzoate
Ubiquinone Q6
ALD
BD
p-HBD
CO2
ADH
BAL
p-HBAL
ADH

Graphic for table of contents