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Molecular and Phenotypic Characterization of *Metschnikowia pulcherrima* Strains from Douro Wine Region

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Abstract: Some non-*Saccharomyces* yeasts, including *Metschnikowia pulcherrima*, have been proposed as selected starters due to their contribution for the overall aroma and chemical profiles of wines. In this work, we aimed to evaluate the genetic and phenotypic diversity of *Metschnikowia pulcherrima* strains isolated from different locations of Douro Wine Region, and to explore their potential as co-adjuncts of *S. cerevisiae* in alcoholic fermentation. For that purpose, a set of 64 *M. pulcherrima* isolates were used. Polymerase chain reaction (PCR) fingerprinting with M13 primers demonstrated to be an efficient tool in intraspecific discrimination of *M. pulcherrima* strains. No significant associations were found between genotypic profiles and either geographical origin or winery. The isolates were screened for their stress resistance ability (ethanol, SO₂, chitosan, copper, H₂O₂, and Grape Juice Medium), aroma-related activities (resistance to 5, 5', 5''-trifluor-D, L-leucine and cerulenin and β-glycosidase, β-lyase and sulfite-reductase activities) as well as other relevant technological proprieties (protease activity and biogenic amines production). *M. pulcherrima* response to the different enological traits evaluated was greatly strain-dependent. The most discriminant features were the ability of the strains to grow in Grape-Juice Medium (GJM) and sulfite-reductase, and their β-lyase and protease activities. The enological potential of a selected *M. pulcherrima* strain in mixed-culture with *S. cerevisiae* was also assessed in natural grape-juice of a local variety, under two nitrogen regimes. *M. pulcherrima* proved to be promising for future industrial application as a co-starter, lowering ethanol, acetic acid and, reported here for the first time, lowering hydrogen sulfide levels in the wines.

Keywords: *Metschnikowia pulcherrima*; wine; stress tolerance; enzymatic activities; mixed-culture; hydrogen sulfide; yeast assimilable nitrogen

1. Introduction

Traditionally winemaking is a natural process conducted by yeast present on grape-musts. Yeast genera frequently found on grapes and in must include *Hanseniaspora*, *Candida*, *Metschnikowia*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Rhodotorula*, *Zygosaccharomyces*, and *Cryptococcus* [1]. These non-*Saccharomyces* yeasts initiate spontaneous alcoholic fermentation of the juice, but they are rapidly surpassed by the growth of *Saccharomyces cerevisiae* as a consequence of their weak SO₂ and ethanol tolerance [1–4]. However, this resident microbiota has been associated with problems in the progression of the fermentation and/or with the formation of off flavors and/or the formation of

undesirable compounds [1,4] such as the biogenic amines [5]. Additionally, these so-called “natural fermentations” results in inconsistent wines from vintage to vintage.

To overcome these issues, the winemaking industry developed inoculation practices with selected *Saccharomyces cerevisiae* strains to limit the involvement of non-*Saccharomyces* yeasts during wine fermentation. However, there has been an increasing recognition that this procedure results in wines lacking the distinctive characteristics and complexity that are associated to the presence of non-*Saccharomyces* strains during fermentation [1,6–9]. Indeed, non-*Saccharomyces* yeasts have long been described as possessing properties that are not found, or at least not so pronounced, in *S. cerevisiae*, such as aroma-related enzymatic activities and interesting enological properties [1,10–13]. These findings have led to the re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking. Accordingly, selection protocols, based on these relevant enological features, have been used to find proper strains among those naturally found in grapes and wines, as previously done to *S. cerevisiae* [14–19]. Over the last years, experiments with mixed starter cultures of *Saccharomyces cerevisiae* and non-*Saccharomyces*, either co-inoculated or inoculated sequentially, have been conducted in order to ally the ability of non-*Saccharomyces* to produce distinct volatile compounds, to the ability of *Saccharomyces* to complete the fermentation process, while minimizing the risk of microbial spoilage [4,8,18–23]. In addition to its contribution to the aromatic profile [24], it was found that these yeasts may have a role on other important wine proprieties by increasing glycerol and mannoprotein contents, reducing volatile acidity and ethanol levels, and/or contributing to color stability [22,25–34]. Presently, there are already several commercial non-*Saccharomyces* strains available for application in the winemaking industry that can be used as a technological solution to specific problems and/or improve sensory characteristics, such as complexity mouth-feel and flavors [35].

Nevertheless, and as seen for *S. cerevisiae* strains, the use of commercial starters could mask the distinctive peculiarities of the wines produced in a given region. Thus, the exploitation of the microbial diversity that exists in the vineyards with the selection of autochthonous yeast strains for use as starters, has been considered an interesting approach to overcome this concern [21,23,27,36–44].

Therefore, in this study we analyzed the genetic and phenotypic diversity of a set of *M. pulcherrima* strains isolated from different locations of Douro Wine Region. In a second stage, one strain of *M. pulcherrima* was selected, based on relevant enological features, to ferment a natural grape must, either in single- or in mixed-culture with a commercial strain of *S. cerevisiae*, under two nitrogen regimes. The results obtained highlighted the biodiversity among the *M. pulcherrima* strains. Additionally, strain Mp39 strain proved to be a good candidate for future application in mixed-culture fermentations helping on the relieve of the some of the challenges of winemaking industry, lowering ethanol, acetic acid and hydrogen sulfide levels in the wines.

2. Materials and Methods

2.1. Yeasts Strains and Maintenance Conditions

Strains used in this study are listed in Table 1. This set included, 64 *Metschnikowia pulcherrima* strains isolated from the three main sub-regions of the Douro Wine Region, Baixo Corgo, Cima Corgo and Douro Superior (Figure 1) and the type strain obtained from the Spanish Type Culture Collection (CECT 11202). The commercial *S. cerevisiae* UCD522 strain used in fermentation trials was supplied by the Enology Culture Collection, Department of Viticulture and Enology, University of California, Davis, CA, USA. The yeasts were routinely maintained at 4 °C on yeast peptone dextrose agar (YPD) slants, containing per liter: 20 g glucose, 10 g peptone, 5 g yeast extract, and 20 g agar, from stocks stored at –80 °C. Immediately before use, the yeasts were transferred to a new slant of YPD and cultured for 24–48 h at 25 °C, unless otherwise stated in the text.

Table 1. Source of isolation of the *M. pulcherrima* strains used in this study.

Douro Subregion	Source	Strain Code
BC	Winery 14	Mp51, Mp53, Mp54, Mp55, Mp56, Mp57, Mp58, Mp59, Mp60, Mp64
	Winery 2	Mp2
	Winery 3	Mp3
	Winery 7	Mp11, Mp14, Mp15
	Winery 8	Mp12, Mp35, Mp40
	Winery 9	Mp13, Mp17, Mp18, Mp38, Mp42, Mp43, Mp44, Mp45, Mp46, Mp47, Mp48, Mp49, Mp50
CC	Winery 1	Mp1
	Winery 4	Mp4
	Winery 6	Mp7, Mp8, Mp9, Mp10, Mp31
	Winery 10	Mp16, Mp30, Mp32, Mp33, Mp34
	Winery 11	Mp19, Mp20, Mp21, Mp22, Mp23, Mp24, Mp36
	Winery 15	Mp52
DS	Winery 16	Mp61, Mp62, Mp63
	Winery 5	Mp5, Mp6
	Winery 12	Mp25, Mp26, Mp27, Mp28, Mp29
	Winery 13	Mp37, Mp39, Mp41
	CECT	Mp CECT11202

BC—Baixo corgo; CC—Cima Corgo; DS—Douro Superior.

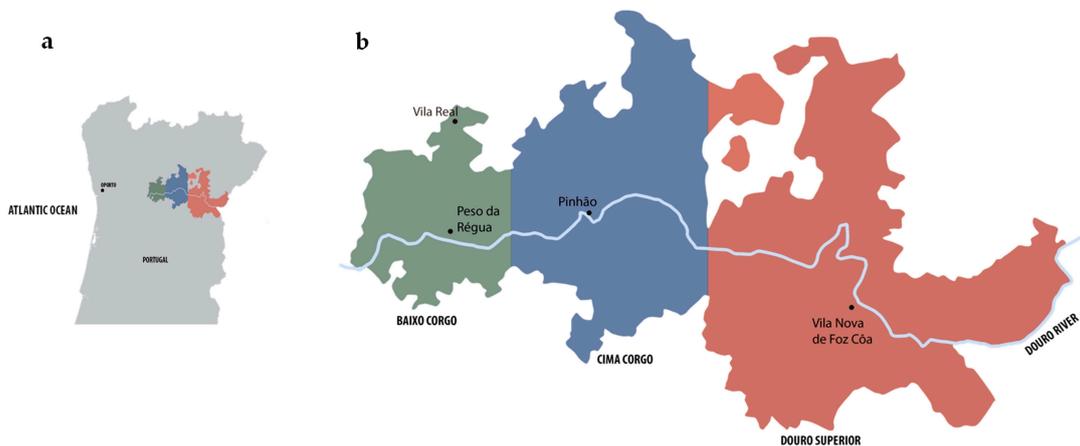


Figure 1. Map of Douro Wine Region: (a) Illustration of the position of the Douro Wine Region within Portugal; (b) Each colored zone represents an individual subregion: Baixo corgo (BC); Cima Corgo (CC); Douro Superior (DS) from which the *M. pulcherrima* strain characterized in this study were isolated.

2.2. Molecular Characterization

To analyze the molecular diversity of the *M. pulcherrima* strains, PCR fingerprinting was performed employing the primers (GTG)₅ and M13.

DNA Extraction and Amplification

DNA extraction was performed following a protocol adapted from Looke et al. [40]. Yeast cells were re-suspended in 200 mM of lithium acetate with 1% of sodium dodecyl sulfate (LiOAc-SDS) and were incubated at 70 °C for 15 min. After that, cells were resuspended in 96% ethanol, centrifuged and again resuspended in 70% ethanol. Precipitated DNA was dissolved in Tris buffer EDTA (ethylenediaminetetraacetic acid) (TE) and frozen at −20 °C for at least 30 min. The supernatant was collected and used for PCR, using an Applied Biosystems thermal cycler, with M13 (5’GAGGGTGGCGTTCT3’) and (GTG)₅ (5’GTGGTGGTGGTGGTG3’) primers. The amplification conditions and sequences for M13 and (GTG)₅ are those described previously by Huey and Hall and Lieckfeldt et al. [41,42], respectively. The fingerprinting profiles were compared by the Pearson’s correlation coefficient and unweighted pair group with arithmetical average (UPGMA) as a clustering method using the BioNumerics software (version 5.0, Applied Maths, Sint-Martens-Laterat, Belgium).

2.3. Phenotypic Characterization

All strains were screened for relevant enological features, including stress resistance and other qualitative traits, such as enzymatic activities, that may impact the chemical composition of wines.

2.3.1. Stress Resistance Assays

For all assays, yeast strains were pre-cultivated in YPD medium and grown until the mid-exponential growth phase in 96-well microtiter plates. Using a stainless steel 96-pin replicator, culture suspensions were inoculated in the appropriate culture medium for stress tolerance and enzymatic activities evaluation. YPD agar plates without a stress agent, was used as control.

Five stress conditions that yeast may encounter during fermentation were tested by spot assay in YPD agar medium, with pH adjusted to 3.5, containing increasing doses of each stress agent, followed by incubation for 2–3 days, at 28 °C. Accordingly, the following concentrations were used: 6%, 9%, or 12% (*v/v*) of ethanol; 1, 2, or 4 mM of sulfur dioxide (SO₂) through adding 5 mL of a stock solution of 15, 30, and 60 mM, respectively; 0.5, 1, or 2 mM of copper, supplied as copper sulfate; 0.1, 0.5, or 1 mg L⁻¹ of chitosan (No Brett inside[®], Lallemand, Proenol, Portugal) and 0.25, 0.5, or 1 mM of H₂O₂ from a 10 mM stock solution.

To identify strains that produce particular flavor compounds, the strains were tested for the capacity to grow in the presence of cerulenin and/or 5, 5', 5''-trifluor-D, L-leucine (TFL). These screens were conducted using agar plates and minimal medium (YNB) supplemented with glucose (2%) and TFL (0.6 mM) [43] or cerulenin (6 μM) [44].

The ability of the *M. pulcherrima* strains to adapt to grape must environment was also evaluated. A chemically-defined Grape-Juice Medium (GJM), similar in composition to typical grape juice [45] was used, with minor modifications. In particular, nitrogen composition consisted of a mixture of amino acids and ammonium in a proportion of 60%:40%, at a final concentration of yeast assimilable nitrogen (YAN) of 267 mg L⁻¹ (GJM₂₆₇). Cell suspensions (3 μL) were inoculated in 100 μL of GJM₂₆₇ media in 96-well microtiter plates, and incubated at 20 °C with orbital agitation (150 rpm). Yeast growth was monitored by optical density (OD) measurement at a wavelength of 630 nm at three time points (0, 24, and 48 h) in a microtiter plate reader model Multiskan Ascent (Thermo Electron Corporation, Vantaa, Finland). Scores for yeast growth were obtained by the determination of the area under the OD/time curve (AUC) using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

2.3.2. Evaluation of Enzymatic Activities

Seven enzymatic activities were evaluated using qualitative assays. The potential ability to produce H₂S was evaluated by growing yeast cells in Biggy agar. This medium uses bismuth as an indicator and the color of the colonies, due to the precipitation of bismuth sulfide, is thought to be correlated with the basal level of activity of sulfite reductase [45,46]. The yeast growth scores were based on the colony color, which ranged from white (scored 0) through brown to near-black (scored 6), after 72 h at 28 °C [47]. The activity of β-lyase was screened using a medium containing 0.1% S-methyl-L-cysteine, 0.01% pyridoxal-5'-phosphate, 1.2% Yeast Carbon Base, and 2% agar [48]. The pH was adjusted to 3.5. Growth of the colonies after 48–72 h indicated the presence of β-lyase activity. Proteolytic activity was evaluated spotting yeast strains on skim milk agar medium. The appearance of a clear zone around the colony, after 72 h at 28 °C, was associated to protease activity [49]. β-glycosidase activity was tested as previously described by Villena et al. [50]. Yeast cells were grown in a medium containing 0.5% cellobiose (4-O-β-D-glucopyranosyl-D-glucose), 0.67% yeast nitrogen base and 2% agar. Growth of the colonies after 48–72 h indicated the presence of β-glycosidase activity. Qualitative detection of histamine, tyramine and putrescine was performed as described by Granchi et al., 2005 [51], with some modifications. Differential culture media containing yeast extract (3%), glucose (1%), the amino acid precursor (2%), histidine, tyrosine or ornithine, and bromocresol purple (0.015 g L⁻¹), at a final pH adjusted to 5.2 were used. The decarboxylation of the amino acid to the corresponding

biogenic amine results in an increase in pH, detected by the change in color of the medium. While histamine and putrescine producing strains were identified by purple coloration, tyramine production was detected by the decolorization of the culture medium.

Each yeast strain was tested for each stress at least twice on three different occasions. Final resistance scores were averaged for the replicates and the stress doses, and divided by the maximum value of the scale, providing a single score for all assays ranging from 0 (no growth/no activity) to 1 (active growth/positive activity) for each strain, and each stress condition as previously reported [47]. The phenotypic profiles of the strains were subjected to cluster analysis and similarity patterns were obtained using Pearson correlation and UPGMA clustering, using SAS JMP 11.0 (JMP, 2011, Cary, NC, USA).

2.4. Single and Mixed-Culture Fermentations

The strain of *M. pulcherrima* Mp39 was used to ferment a natural grape must of Tinta Roriz, one of the five Douro Valley indigenous grape varieties recommended for production of quality table and Port wines, either in single- or mixed-culture with *S. cerevisiae* UCD522, under two nitrogen regimes.

2.4.1. Grape-Juice Preparation

Natural grape-juice was obtained by pressing Tinta Roriz grapes; after homogenization, the juice was clarified by centrifugation at $12,734 \times g$ for 10 min. Then the following parameters were determined: °Brix, pH, titrable acidity and yeast assimilable nitrogen (YAN). Grape-must was split into two lots, one of which was supplemented with 1.0 g L^{-1} of di-ammonium phosphate (DAP), resulting in a final concentration of $280 \text{ mg YAN L}^{-1}$, while the other lot remained as a control ($73.5 \text{ mg YAN L}^{-1}$). Supplemented and non-supplemented grape-juices were transferred to sterile 500 mL-flasks and held at $70 \text{ }^\circ\text{C}$ for 10 min, to eliminate most of indigenous yeasts and to assure dominance of the tested strains, then immediately cooled on ice and stored at $-20 \text{ }^\circ\text{C}$. No sulfur dioxide was added. The initial pH of grape-juice was 3.06 and the concentration of sugars was 20° Brix.

2.4.2. Inocula Preparation and Fermentation Conditions

Alcoholic fermentations were carried out either with single culture fermentations of *M. pulcherrima*, or *S. cerevisiae*, and with mixed-cultures of *S. cerevisiae* with *M. pulcherrima*, under two nitrogen regimes. Uninoculated grape juice was used as control. Starter cultures of each strain were prepared by growing the yeast overnight in 100 mL-flasks, containing 50 mL of synthetic grape-juice medium with $267 \text{ mg YAN L}^{-1}$, supplied as Di-ammonium phosphate (DAP). The flasks were incubated at $25 \text{ }^\circ\text{C}$ in an orbital shaker set at 150 rpm. Each yeast species was inoculated at a cell count of 10^6 colony forming units (CFU) mL^{-1} . The fermentations were conducted in 500 mL-flasks filled to 2/3 of their volume fitted with a side-arm port sealed with a rubber septum to allow anaerobic sampling, and were maintained at $20 \text{ }^\circ\text{C}$ in an orbital shaker set at 120 rpm. Fermentations were monitored daily by weight loss as an estimate of CO_2 production. Aseptic sampling was done daily for assessing fermentation and growth parameters using a syringe-type system, allowing the fermentation gases to escape through a glass tube connected to a two-way valve by Teflon tubing, which was connected to a fermentation lock and an H_2S trap, as previously described [52]. Every 24 h, all flasks were removed from the shaker, weighed, disconnected from the trap, and connected to a new trap. Prior to sampling, the flasks were stirred to ensure homogeneity. Fermentations were allowed to proceed until no further weight loss. As fermentations conducted by single-cultures of *M. pulcherrima* were not expected to be complete, they were stopped at the same time as that for the mixed-culture fermentations. At this time point the viable yeast cell number, culture dry weight and residual YAN levels were measured. Each set of fermentations was repeated at least three times and reported data represent mean values of the replicated experiments.

2.4.3. Determination of Yeast Growth and Fermentation Parameters

Yeast growth was followed by periodic counting of CFU in YPD agar plates after incubation at 30 °C, for 48 h. Colonies of *S. cerevisiae* UCD522 in mixed cultures were distinguished from those of *M. pulcherrima* Mp39 by the development of colonies of cream color with hint of red/red brown by the latter. Culture dry weight was determined in samples of 3 × 50 mL at the end of fermentation, which were centrifuged in pre-weighed tubes for 10 min at 2300 × g, washed twice with sterile deionized water, dried for 24 h at 100 °C, and stored in a desiccator before weighing. Maximum fermentation rate was determined from the slope of the linear dependence of the steepest decline in weight (g) at the corresponding time points (h).

2.5. Analytical Determinations

Yeast assimilable nitrogen (YAN) was determined by the formol method as published elsewhere [53]. Total SO₂, pH, titratable acidity, volatile acidity and ethanol content were determined following the standard methods. All physicochemical analyses were performed prior to and at the end of alcoholic fermentations. The amount of H₂S released by yeast cultures was determined colorimetrically following the selective collection of fermentation gases with a modified fermentation lock and sulfide-trapping system [52]. Sulfide concentration was calculated from a calibration curve established from known quantities of sulfide (0–12 µg L⁻¹).

2.6. Statistical Analysis

Chi-square tests were run to determine if there was a statistical association between geographical origin and genotypic fingerprinting. The effects of inoculation strategy and initial YAN concentration on fermentation kinetics and on physicochemical parameters of the final wines were examined through a two-way analysis of variance (ANOVA) and Student's *t* test for paired comparisons, using SAS JMP 11.0 (JMP, 2011, Cary, NC, USA). For all analysis *p* < 0.05 was considered statistically significant.

3. Results and Discussion

Non-*Saccharomyces* are currently recognized as potential adjuncts to *S. cerevisiae*, and selection programs, as done in the past for *S. cerevisiae*, must be implemented among native yeasts before exploiting them as blends of starters to obtain wines with specific regional characteristics and styles [54]. To our knowledge this is the first work reporting the biodiversity of *Metschnikowia pulcherrima* strains taking inconsideration not only relevant enzymatic activities but also their ability to grow and persist during alcoholic fermentation.

3.1. Molecular Characterization

Fingerprinting with the primer M13 resulted in a higher number of distinct profiles allowing a better strain discrimination than DNA polymorphisms obtained for (GTG)₅. Indeed, at a similarity limit of 60%, the former enabled the separation of the yeast strains into nine distinct clusters while using the latter *M. pulcherrima* strains grouped in only three clusters. This controverts reports by Libkind et al. [55] about the usefulness of M13 primer-based fingerprinting for the typing of closely related yeast strains, as it is supposed to amplify more conserved regions of DNA. Our results suggest that this primer could be an efficient tool in intraspecific discrimination of *M. pulcherrima* strains in future studies. In Figure 2 is depicted the dendrogram derived from the combined PCR fingerprints obtained using each of the primers. The cluster analysis showed some heterogeneity among these *M. pulcherrima* isolates. At the similarity level of 50% we could recognize three major clusters and a single strain cluster represented by Mp32. Overall, isolates obtained from the same winery were found among the three clusters although we observed some grouping of strains with profile similarities above 90%. For instance, strains Mp5 and Mp6 were both isolated from winery nine and could possibly be the same strain. On the other hand, it is worth noting, that strains Mp36, Mp37 and Mp38, which

were isolated from the different sub-regions within the Douro Wine Region, presented genotypic profiles that were very similar. Accordingly, no significant association ($p > 0.05$; chi-square test) was found between genotypic profile and either geographical origin or winery.

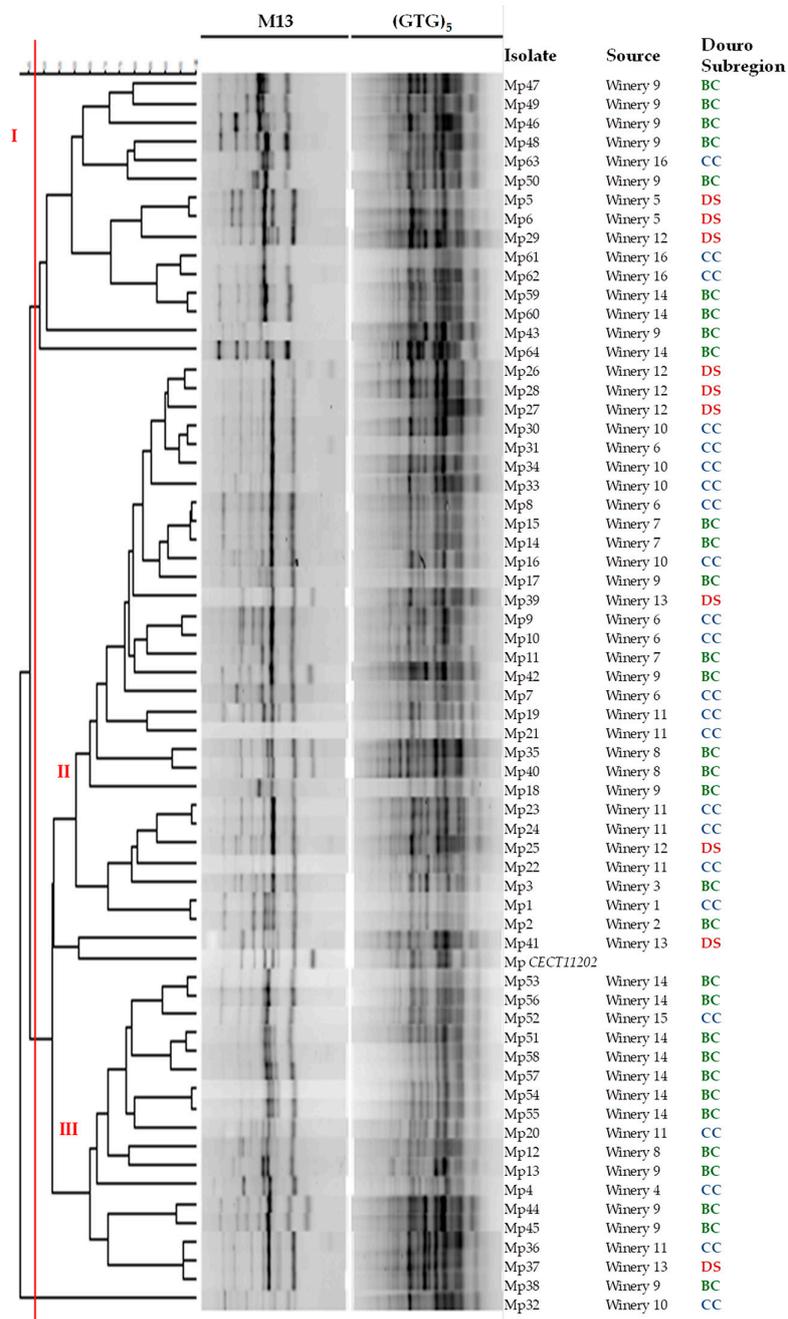


Figure 2. Genotypic variability of the 65 *M. pulcherrima* yeast strains, including the type strain (CECT11202). Dendrograms obtained by composite hierarchical analysis of polymerase chain reaction (PCR) (GTG)₅ and M13 patterns using Pearson’s correlation coefficient and the UPGMA clustering method for the isolates. BC—Baixo corgo; CC—Cima Corgo; DS—Douro Superior.

3.2. Phenotypic Characterization

Yeast response to important enological traits under study was greatly strain-dependent. The phenotypic relatedness of the *M. pulcherrima* strains was established using cluster analysis. In this line, the phenotypic profiles based on the final scores for each strain in each condition tested (Table S1)

were organized using the Pearson correlation and UPGMA clustering. Accordingly, and as shown in the Figure 3, the strains were separated in five distinct clusters.

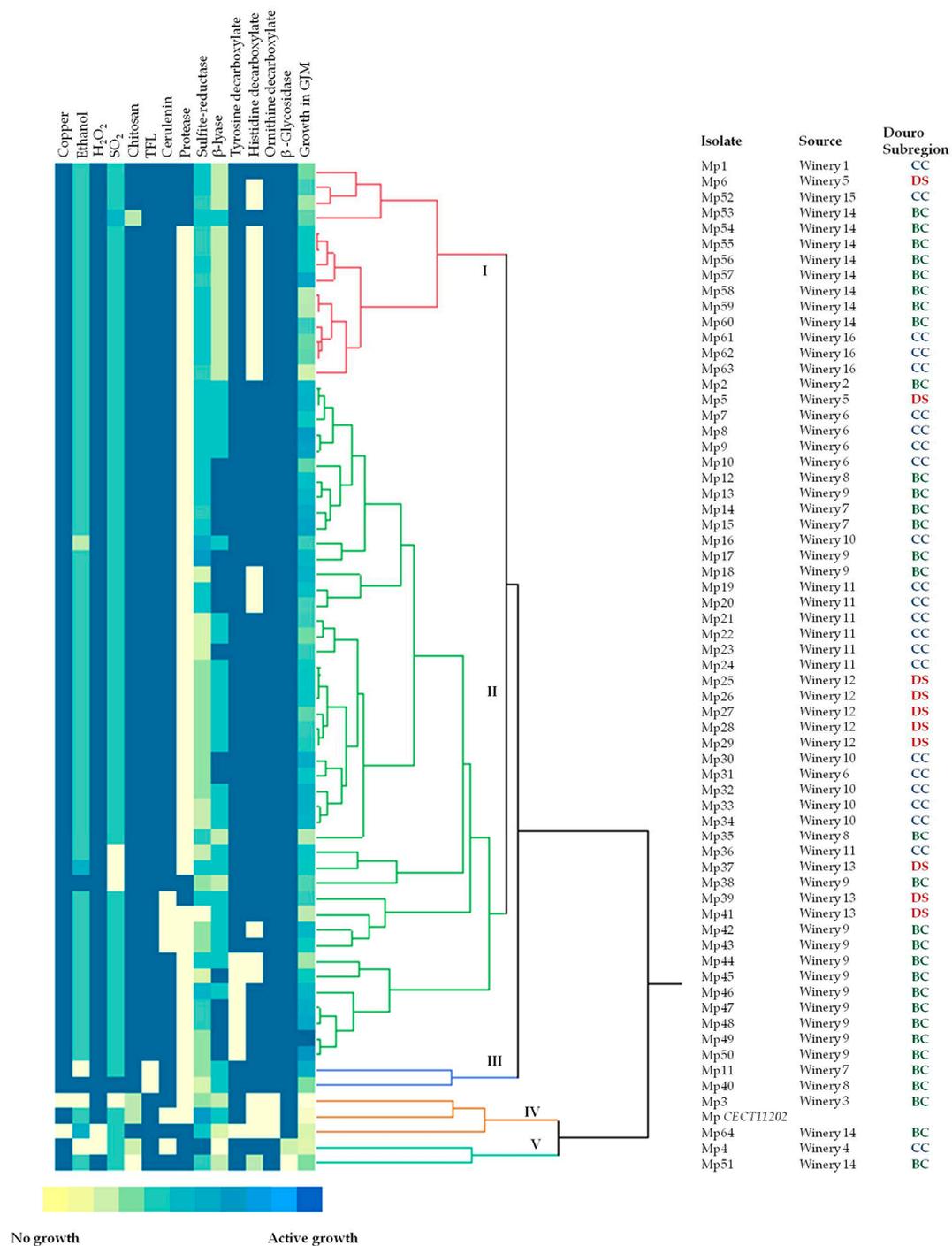


Figure 3. Phenotypic variation in the 65 *M. pulcherrima* yeast strains including the type strain (CECT11202). Each cluster is marked with different colors. Each row on the plot represents a different strain, and each column indicates a particular environment. The colored boxes represent the average growth score of each strain in each environment. BC—Baixo corgo; CC—Cima Corgo; DS—Douro Superior.

Fifty-eight out of the 65 strains clustered in two major groups, clusters I and II, while the remaining seven were distributed in three minor clusters (clusters III–V). Based on the mean scores obtained

for each test in each cluster (Figure S1), overall, the most discriminant features were the ability of the strains to grow in GJM₂₆₇ and sulfite-reductase, β -lyase and protease activities. In particular, strains contained in cluster I were distinct from those included in cluster II due to higher sulfite-reductase and protease activities and lower β -lyase and histidine decarboxylase activities. All strains sensitive to TFL and all strains lacking ornithine decarboxylase activity were included in clusters III and IV, respectively. The remaining cluster encompassed the strains with higher β -glycosidase activity and lower chitosan tolerance. As seen previously for the genotypic profiles of these strains, no correlation was found between the source of isolation and the phenotypic patterns (chi-test, $p > 0.05$). This observation was already described for *S. cerevisiae* strains [47,56], *C. zemplinina* [21] and other non-*Saccharomyces* strains of the same species from different origins [48].

Some interesting discrepancies in the phenotypic profiles were observed among and inside different clusters of the dendrogram obtained with the fingerprinting composite. Strains with similar PCR-fingerprinting patterns grouped differently in terms of phenotypic traits. For example, Mp35 and Mp40, included in cluster II with similarity above 90% (Figure 2) displayed significant differences in ethanol and TFL resistance as well as in sulfite-reductase activity, being included in Clusters II and III, respectively (Figure 3). On the other hand, strains Mp19 and Mp20 with highly similar phenotypic profiles (cluster II, Figure 3) showed considerable differences in the genotypic patterns, being separated in clusters II and III (Figure 2). Nevertheless, some strains were shown to be very similar by both dendrograms. This was most striking in the case of the strains Mp36, Mp37 and Mp38 that were isolated from different sub-regions. This observation suggests that, besides geographical distance, other factors such as grape variety and anthropogenic aspects are involved in strain distribution.

Yeast growth profiles in a synthetic grape-juice medium were investigated, and were linked to the ability of *M. pulcherrima* strains to adjust to winemaking environment. As previously mentioned, the 65 strains showed a great diversity in their ability to grow in GJM₂₆₇. The highest AUC value was obtained for the strain Mp44, while Mp3 displayed the worst performance, with impaired growth in 70%. This feature is of particular interest as it is associated to the strain ability to impose on the others in a competitive environment like grape-must, and it will allow a prolonged longevity during the fermentative process, increasing its contribution to final wine composition.

Most of the *M. pulcherrima* isolates managed to tolerate at least 6% *v/v* ethanol concentration (62 out of 65 strains) while at 9% *v/v* ethanol, only four strains, Mp38, Mp39, Mp40 and Mp64, displayed active growth. Any of the isolates was able to grow in the presence of the maximum concentration tested (12% *v/v*). This is the first time that this high ethanol tolerance has been reported in *M. pulcherrima* strains. Indeed, they have been characterized as low ethanol tolerant strains (3–5% *v/v*) either based on the loss of viability during fermentations with *S. cerevisiae* and/or by the amount of ethanol produced when in single culture [3,4,27–29,57].

The ability of wine strains to tolerate SO₂ is an important characteristic in commercial wine fermentations, as its application is mandatory for controlling the growth of spoilage microorganisms, as well as to prevent oxidation due to its antioxidant and antioxidasic proprieties. Herein, all *M. pulcherrima* strains were able to grow in media containing up to 2 mM of SO₂. Most noticeable, strains Mp6 and Mp40 were able to resist to the highest concentration tested (4 mM). Similar observations have been reported, using different strains and methodologies [4,8,57]. These findings contrast with the concept that non-*Saccharomyces* yeasts, in general, are sensitive to the common doses of SO₂ used in winemaking, and contest one of the fundamental reasons for using SO₂ in winemaking [2]. Moreover, due to the increasing interest in more organic wines, there is a tendency in the regulations for limiting the use of SO₂, which have led to the search for alternative compounds to replace it [58]. Among these compounds there is chitosan, whose applicability as a microbial control agent against wine related microorganisms was recently studied [59]. The authors found that chitosan was able to inhibit the growth of the non-*Saccharomyces* yeasts *Hanseniaspora uvarum*, *ZygoSaccharomyces bailii* and *Brettanomyces bruxellensis* with Minimum Inhibitory Concentration (MIC) values of 0.4, 0.4, and 0.2 g L⁻¹, respectively. However, the efficacy of chitosan was decreased under winemaking

conditions. Our results show that the majority of *M. pulcherrima* strains tolerated the highest chitosan concentration tested (1 g L^{-1}), being yeast growth compromised only for four yeast natural isolates, Mp3, Mp4, Mp6 and Mp51. Taken together, these findings raise questions about the effectiveness of chitosan in controlling indigenous yeasts and encourage further studies to be performed before considering it as an alternative to SO_2 .

The application of copper is one of the oldest methods used in viticulture against fungal diseases of the vines, and its use has been intensified in the last years, as it is one of the few tools that can be used by organic vine growers [60]. Consequently, the selection of resistant strains is crucial as there is a risk of high levels of copper residues in grape-must [61]. Herein, all isolates tolerated the maximum concentration of copper sulfate tested (2 mM), except two isolates (Mp3 and Mp64). The improved resistance of *S. cerevisiae* strains to copper [62,63] and to sulfites [64,65] is considered as potential domestication-related trait as a consequence of environmental adaptation to these two chemical compounds used in vineyards and during winemaking. Given that strains from different environments were not included in this study we can only speculate that *M. pulcherrima* strains might have experienced a similar process of adaptive evolution.

Resistance to oxidative stress has been pointed has an advantage for *S. cerevisiae* during fermentation as yeast cells produce reactive oxygen species in a high-sugar medium [66,67]. The strains that better manage oxidative stress from either yeast respiratory metabolism or the presence of H_2O_2 produced during alcoholic fermentation will likely perform better during fermentation [68]. In this study, all isolates of *M. pulcherrima* were able to grow under the maximum level of H_2O_2 tested (Figure 2 and Table S1). Our results are consistent with those found by Furlani et al. [16] that evaluated H_2O_2 tolerance in this and other non-*Saccharomyces* species. The authors suggested that this feature could be associated with the catalase activity expressed by these yeasts.

Yeast selection criteria for application in winemaking also include metabolic traits, which affect the organoleptic properties of wines. In our work, besides the well-documented importance of enzymatic potential of yeast, we also evaluated strains resistance to either TFL or cerulenin. Their ability to grow in these media has been associated to its potential to produce flavoring compounds, higher alcohols and esters, respectively [69,70]. Accordingly, cerulenin and TFL resistance has been evaluated for the characterization of *S. cerevisiae* strains for wine [44,47] and cachaça [43] fermentations. Common features of these works include the weak resistance of *S. cerevisiae* to TFL and cerulenin and the low frequency of resistant strains to both compounds. Similar strategies have not, to date, been applied in non-*Saccharomyces* yeast selection programs. In this study, 90% of the *M. pulcherrima* strains showed high resistance to both compounds. Only two and six isolates showed diminished growth in the presence of TFL or cerulenin, respectively (Table S1).

Also, enzymatic activities associated with the potential liberation of desirable or undesirable aromas for winemaking were evaluated. All *M. pulcherrima* isolates, except Mp4 and Mp51, displayed remarkable β -glycosidase activity. Our results are in line with previous reports that verified that *M. pulcherrima* is one of the non-*Saccharomyces* species with higher potential to release grape-derived terpenes from their glycosylated precursors [48,71]. Also directly related to varietal aroma enrichment is β -lyase activity, which is involved in the release of volatile thiols from cysteinylated precursors [12]. All strains presented β -lyase activity at different extent, with 17 strains exhibiting a remarkable activity (Figure 3, Table S1). Although the knowledge on the potential of non-*Saccharomyces* yeast to release volatile-thiols in wine is very limited, there are evidences that this feature is strain-dependent [48,72]. Regarding the sulfite reductase activity, the strains exhibited variable colony color intensity on Biggy agar, demonstrating different potential to produce H_2S [46,73]. Based on the level of darkness of the colonies color, the strains were classified as high (5 strains), moderate (53 strains) and as low H_2S producers (7 strains). Similar results have been recently found by Aponte and Blaiotta [57] in the characterization of isolates from Aglianico grapes. This diversity among *M. pulcherrima* strains conflicts with the results of Belda et al. [48] that reported very low or absent sulfite-reductase activity

in *Metschnikowia* spp. isolates. Taken together, our results underlie the potential of *M. pulcherrima* strains to impact wine sensory profiles.

Selection of strains with protease activity might also be beneficial as it may improve wine processing, through the facilitation of juice extraction and clarification and wine filtration [74]. Also, the degradation of grape juice proteins could be a source of nitrogen for yeast growth [1]. Several studies have shown that some non-*Saccharomyces* species, have an interesting potential in this regard [75,76]. In this study, we found only nine *M. pulcherrima* isolates exhibiting proteolytic activity.

Metabolic activity of yeasts may also give rise to the formation of biogenic amines (BAs), which are toxic to humans. Therefore, it is of particular importance to assess yeast potential to produce these compounds contributing to the safeguarding of consumer health [58]. Two studies on biogenic amines production by five different wine yeast species [5,51] clearly showed that this attribute is species- and strain dependent. *Brettanomyces bruxellensis* was found to be the highest producer in both studies, followed by *S. cerevisiae*, *M. pulcherrima*, *Kloeckera apiculata* and *Candida stellata*. In this work, the potential ability of *M. pulcherrima* strains to produce histamine, tyramine and putrescine was highly variable. Ornithine decarboxylase activity was found in 62 strains, 57 and 43 strains exhibited tyrosine and histidine decarboxylase activities, respectively. It should be noted that the methodology used herein is merely qualitative and further studies should be performed to quantify the potential contribution of these yeast to the levels of BAs in the wine and to evaluate the associated risks for the consumer.

3.3. *S. cerevisiae* and *M. pulcherrima* Single and Mixed Fermentations

The isolate Mp39 was selected to conduct fermentations, either in single- or in mixed-culture with the commercial strain *S. cerevisiae* UCD522, based on the high tolerance to ethanol and SO₂ levels (up to 9% (v/v) and 2 mM, respectively), the good growth performance in GJM₂₆₇, high β-glycosidase, β-lyase and protease activities, and low ability to produce H₂S. Given the known effect of nitrogen availability on *S. cerevisiae* performance during alcoholic fermentation either in single [47,52,77,78] or in mixed-culture with non-*Saccharomyces* yeast [22,79], the fermentation trials were conducted under two different nitrogen regimes.

A global view of yeast growth and fermentation profiles is presented in Figure 4. *M. pulcherrima* Mp39 behaved similarly under both initial nitrogen concentrations tested, reaching maximum cell population of 1–3 × 10⁷ CFU/mL in single, that were sustained until the fermentations were stopped. As described for *S. cerevisiae* [52,80], nitrogen seems not to be a limiting factor for Mp39 growth, since no significant differences were detected in growth rate and in biomass produced under both nitrogen regimes (Table 2). Nevertheless, significant differences were found in maximum fermentation rate of the *M. pulcherrima* strain used in both conditions.

Table 2. Overview of growth and fermentation parameters obtained in the fermentations conducted by *S. cerevisiae* UCD522 and *M. pulcherrima* Mp39 in single- or in mixed-cultures, containing an initial nitrogen concentration of 73.5 or 280 mg YAN L⁻¹, GJ and GJ + DAP, respectively. Results are mean values from triplicate experiments with their standard deviation.

Grape Juice	Starter Culture	Maximum Fermentation Rate (g h ⁻¹)	Specific Growth Rate (h ⁻¹)		DW (g L ⁻¹)
			Sc UCD522	Mp39	
GJ	Sc UCD522	0.206 ± 0.009 ^c	0.094 ± 0.021 ^a		2.64 ± 0.03 ^b
	Sc UCD522 + Mp39	0.167 ± 0.006 ^d	0.092 ± 0.008 ^{ab}	0.056 ± 0.004 ^{cd}	2.18 ± 0.76 ^{b,c}
	Mp39	0.050 ± 0.006 ^f		0.067 ± 0.014 ^{b,c,d}	1.32 ± 0.00 ^c
GJ + DAP	Sc UCD522	0.494 ± 0.009 ^a	0.075 ± 0.009 ^{ab,c}		5.07 ± 0.12 ^a
	Sc UCD522 + Mp39	0.467 ± 0.017 ^b	0.060 ± 0.002 ^{cd}	0.044 ± 0.009 ^d	2.90 ± 0.26 ^b
	Mp39	0.083 ± 0.001 ^e		0.080 ± 0.003 ^{ab,c}	1.53 ± 0.29 ^c

Values in the same column with different superscript letters are significantly different (*p* < 0.05).

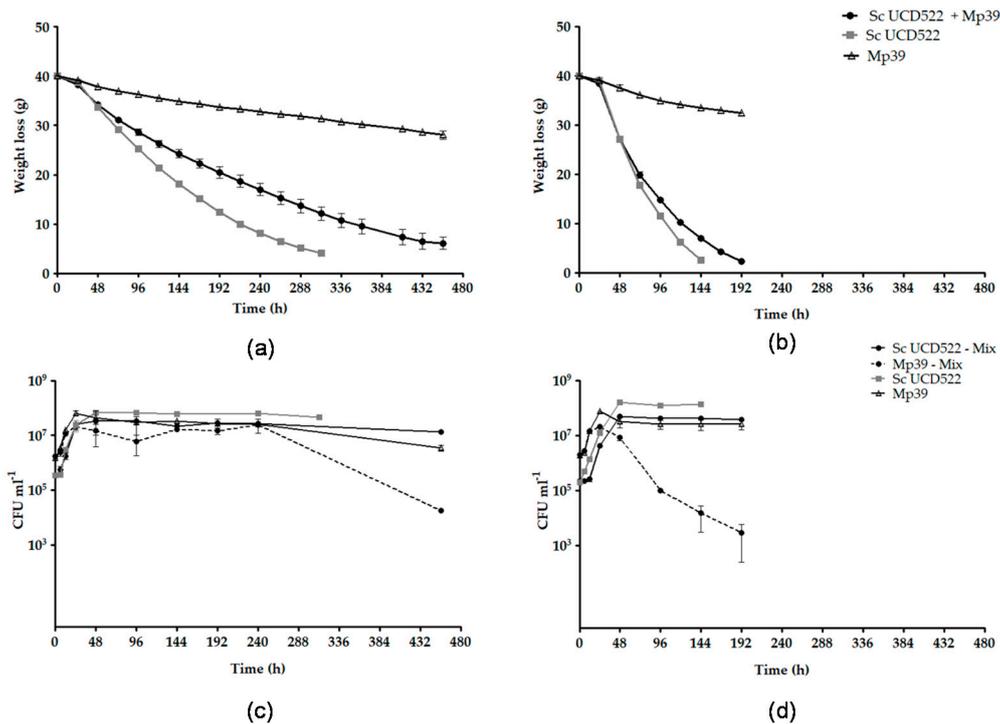


Figure 4. Fermentation kinetics (a,b) and growth profiles (c,d) of single- or mixed-cultures of *S. cerevisiae* UCD522 and *M. pulcherrima* Mp39 in natural grape-juice obtained from grapes of Tinta Roriz at 20 °C, containing an initial nitrogen level of 73.5 mg YAN L⁻¹ (a,c) or supplemented with DAP up to a final concentration of 280 mg YAN L⁻¹ (b,d). Data points are mean values from triplicate experiments with their standard deviation.

On the other hand, while in mixed-culture fermentations, Mp39 was able to attain similar cell count of about 10⁷ but started to decrease from day 2 or day 10 onwards, in the fermentations of musts with or without DAP supplementation, respectively (Figure 4c,d). This loss of viability of *M. pulcherrima* in the first days of mixed-culture fermentations are in line with previous reports [26,81,82], and has been associated to the toxic effect of ethanol produced by *S. cerevisiae*. The results obtained in the phenotypic characterization (See Figure 3 and Table S1), showed that this strain is able to resist at least up to 9% (v/v). Accordingly, the Mp39 strain was able to persist until the end of mixed-culture fermentations, with counts spanning from 10³ to 10⁴ CFU mL⁻¹ (Figure 4c,d), where high ethanol concentrations, up to 10.75% (v/v) were found (Table 3). Another explanation could be the low redox potential in the fermented media since the CO₂ production by *S. cerevisiae* creates anaerobic conditions. In accordance, Hansen et al. [83] have reported that oxygen availability increased the survival time of other non-*Saccharomyces* yeasts, *Torulopsis delbrueckii* and *Kluyveromyces thermotolerans*.

The strain Mp39 had a negative effect on *S. cerevisiae* growth and fermentative performance, as seen by the lower fermentation rates (Table 2) and longer fermentations (Table 3), in both nitrogen regimes. Indeed, a 3.5-fold decrease in *S. cerevisiae* maximum cell population was detected when in co-culture with Mp39 compared with the maximum cell population achieved in single cultures (Figure 4c,d, Table 2). An inhibitory effect of *M. pulcherrima* strains against other wine related yeast, namely *Brettanomyces*, *Hanseniaspora* and *Pichia*, has been described yet, without apparent effect on *S. cerevisiae* [81]. This activity seems to be related to the diffusion of pulcherriminic acid as precursor of pulcherrimin, which depletes the free iron in the medium thus generating an environment unsuitable for microorganisms requiring such element for the growth [84]. Considering that this effect could be strain dependent, this phenomenon could also explain the compromised growth of *S. cerevisiae* UCD522 when in co-culture with Mp39 (Figure 4c,d and Table 2). On the other hand, competition for other nutrients could also account for this observation, as seen in mixed-culture fermentations

with a strain of *Hanseniaspora guilliermondii* [22]. Since no significant differences were detected in the amounts of YAN in the wines obtained by single-cultures of *S. cerevisiae* and by mixed-culture with *M. pulcherrima*, irrespective of the initial nitrogen regime, we may suggest that Mp39 does not seem to compete with *S. cerevisiae*, at least, for nitrogen compounds. The possible interactions of Mp39 with *S. cerevisiae* deserve further investigation.

The presence of *M. pulcherrima* also prompted a great impact on the physicochemical composition of the final wines. Overall, and at least taking into account the parameters analyzed herein, this strain established a good interaction with *S. cerevisiae* by significantly decreasing the levels of volatile acidity, ethanol and H₂S (Table 3). Indeed, the wines obtained by mixed cultures showed a significant decrease in ethanol levels compared with those obtained by single cultures of *S. cerevisiae*, particularly when higher initial levels of YAN were available (Table 3). Our results are in line with other studies that also found ethanol content reduction in fermentations conducted with sequential inoculation of *M. pulcherrima* and *S. cerevisiae* strains [27–29,85]. This phenomenon has been associated with the respiratory metabolism of *M. pulcherrima* strains, which under suitable aeration conditions, are able to respire 40 to 100% of the sugar consumed, with consequent lowering of final ethanol yields [27].

The significant decrease in volatile acidity in wines obtained in the mixed-culture fermentations of non-supplemented grape musts (Table 3) should be highlighted, since this parameter is a negative fermentation by-product, which depreciates wine quality. This clear advantage of the use of mixed cultures over pure cultures of *S. cerevisiae* in the control of volatile acidity was also observed in other studies using *M. pulcherrima* strains [4,29], or other non-*Saccharomyces* yeast species [21]. Interestingly, Benito et al. [85] did not find significant differences in the levels of acetic acid among wines obtained with *S. cerevisiae* with or without prior inoculation with a commercially available *M. pulcherrima* strain.

In line with the results obtained in the phenotypic characterization (See Figure 3 and Table S1), it was clear that the low ability of the isolate Mp39 to produce hydrogen sulfide; single-cultures of *M. pulcherrima* yielded almost 10-fold less than single cultures of *S. cerevisiae*, irrespective of the initial nitrogen content (Table 3). A strong positive effect on the decrease of H₂S formation was seen in mixed culture fermentations, particularly when DAP was added to the must, where a decrease of 8.6-fold was observed (Table 3). Previously, Lage et al. [22] have observed the same effect using a co-culture of *H. guilliermondii* and the same commercial strain of *S. cerevisiae* used herein. Later, Barbosa et al. [79] showed that the presence of the non-*Saccharomyces* strain of *H. guilliermondii* had an evident regulatory control of *S. cerevisiae* genes that impact H₂S liberation during wine fermentation [86–88]. In this line, we could only speculate that Mp39 could have a similar effect on the transcriptome of *S. cerevisiae*. Other possible explanations could be related with sulfur containing amino acids, whose availability regulates the activation of the sulfate reduction pathway (SRS) [89]. As it was seen in our phenotypic screening, Mp39 exhibited proteolytic activity, which could have led to an enrichment of the media in nitrogen compounds, repressing SRS pathway with the consequent reduction of sulfide production [45]. Even so, the impact of *M. pulcherrima* Mp39 on H₂S production remains speculative and deserves further investigation.

Table 3. Overview of chemical composition of the wines obtained in the fermentations conducted by *S. cerevisiae* UCD522 and *M. pulcherrima* Mp39 in single- or in mixed-cultures, containing an initial nitrogen concentration of 73.5 or 280 mg YAN L⁻¹, GJ and GJ + DAP, respectively. Results are mean values from triplicate experiments with their standard deviation.

Grape Juice	Starter Culture	Fermentation Length (h)	pH	Volatile Acidity (g L ⁻¹) *	Total SO ₂ (mg L ⁻¹)	Titrateable Acidity (g L ⁻¹) **	Ethanol (% v/v)	Total H ₂ S (µg L ⁻¹)	Final YAN (mg L ⁻¹)
GJ	Sc UCD522	312	3.11 ± 0.01 ^a	0.29 ± 0.02 ^a	8.19 ± 0.72 ^c	7.63 ± 0.13 ^a	11.40 ± 0.00 ^{a,b}	301.86 ± 21.18 ^b	8.75 ± 2.47 ^b
	Sc UCD522 + Mp39	456	2.98 ± 0.18 ^{a,b}	0.18 ± 0.04 ^b	10.50 ± 1.08 ^{b,c}	7.37 ± 0.40 ^a	10.60 ± 0.42 ^b	278.75 ± 82.02 ^b	1.75 ± 2.47 ^b
	Mp39	-	2.92 ± 0.01 ^{a,b}	0.18 ± 0.00 ^b	10.24 ± 0.00 ^{b,c}	7.76 ± 0.05 ^a	3.75 ± 0.64 ^c	40.35 ± 4.38 ^c	10.50 ± 0.00 ^b
GJ + DAP	Sc UCD522	144	2.91 ± 0.02 ^{a,b}	0.09 ± 0.00 ^c	9.98 ± 1.81 ^{b,c}	8.55 ± 0.42 ^a	12.10 ± 0.28 ^a	397.11 ± 13.17 ^a	8.75 ± 2.47 ^b
	Sc UCD522 + Mp39	192	2.90 ± 0.06 ^b	0.17 ± 0.02 ^b	11.78 ± 0.73 ^{a,b}	7.80 ± 0.21 ^a	10.75 ± 0.07 ^b	46.51 ± 9.19 ^c	10.50 ± 4.95 ^b
	Mp39	-	2.94 ± 0.07 ^{a,b}	0.11 ± 0.02 ^c	13.32 ± 1.45 ^a	9.10 ± 1.83 ^a	3.50 ± 0.01 ^c	25.03 ± 8.92 ^c	126.00 ± 29.69 ^a

GJ—Grape-juice; DAP—di-ammonium phosphate; YAN—yeast assimilable nitrogen. * As g L⁻¹ acetic acid. ** As g L⁻¹ tartaric acid. Values in the same column with different superscript letters are significantly different (*p* < 0.05).

4. Conclusions

This work presents, for the first time, a more in-depth characterization of *M. pulcherrima* strains, demonstrating the potential usefulness of this yeast species for winemaking applications. Collectively, the characterization of the *M. pulcherrima* strains highlighted variability either in genotypic or in phenotypic patterns, emphasizing intraspecies variability and challenging our concept of species typicity. Taken together, the findings here reported highlight the importance of the characterization of the autochthonous microbiota and the integration of non-*Saccharomyces* strains in the design of resources for sustainable and rational advances in the wine sector, being the role of *M. pulcherrima* promising in the pursue of wines with better composition. In particular the strain Mp39 represents a good promise for contributing to wine quality and helping on the relieve of the challenge of winemaking industry, such as lowering ethanol, acetic acid, and hydrogen sulfide levels in the wines. Further studies are needed to elucidate the impact of this strain on wine volatile and non-volatile composition.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/2311-5637/4/1/8/s1.

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