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Genetic Improvement of wine yeasts for opposite adsorption activity of phenolics and ochratoxin A during red winemaking

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ABSTRACT

The aim of this research was to acquire new strains of *Saccharomyces cerevisiae* exhibiting opposite characteristics of cell wall adsorption: very high adsorption activity toward the ochratoxin A, very low adsorption activity toward the pigmented phenolic compounds contained in musts from black grapes. For this purpose, starting from 313 strains of *Saccharomyces cerevisiae*, 12 strains were pre-selected and used to obtain 27 intraspecific hybrids. Eleven crosses out of 27 were validated as hybrids; the best five hybrids were used in guided winemaking at four Calabrian wineries. The employed experimental protocol has allowed to select yeast strains for their different adsorption activity, improving the strains by spore clone selection and construction of intraspecific hybrids. These results suggest an efficacious way to improve the characteristics of interest in wine yeasts.

KEYWORDS

Adsorption; clonal selection; hybrids; ochratoxin A; phenolics; wine making; yeasts

1. Introduction

The wine yeast selection constantly evolves and, consequently, new traits are proposed in order to gain new specific wine characteristics (Ďurčanská et al. 2019). One of these traits, the parietal adsorption activity, is notably different from yeast to yeast. These differences are related to structural characteristics and chemical composition of the outermost layer of cell wall and generate numerous enological effects (Caridi 2006). The ability to adsorb in winemaking unquestionably harmful substances, such as ochratoxin A (OTA) or commonly useful substances such as pigmented polyphenolic compounds is a strain-dependent trait (Caridi and Sidari 2012). So, while excluding the strains of *Saccharomyces cerevisiae* able to adsorb both these compounds, it may be possible to select, with specific protocols, yeast strains able to mainly adsorb OTA. In fact, while the strains with high adsorbing activity are generally useful to produce white wines, the strains with high adsorbing activity

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toward the OTA and low adsorbing activity toward the pigmented polyphenolic compounds may be preferable to produce red wines. Moreover, it was reported that the grape must composition affects the yeast adsorption ability toward colored polyphenols and astringency of wines (Rinaldi et al. 2016; Sidari and Caridi 2016). The greater or lesser phenolic adsorption on yeast cell wall influences concentration and composition of phenolics in wine. Significant correlations between yeast strain used for winemaking and phenolic content in wine were reported, demonstrating that strain behavior can somewhat modify chromatic properties, phenolic profile and antioxidant power of wines (Caridi et al. 2004, 2017a, 2015; Samoticha et al. 2019).

Grape must can contain different amounts of OTA, often due to the growth of mycotoxin-producing molds at the end of grape ripening. Climatic and geographic differences influence mold growth and OTA contamination of grapes. Nowadays, the European legal limit for OTA concentration in wine is of 2 μg/kg (Benito 2019). In Europe, higher OTA levels were detected in wines originating from southern areas with typically warmer climates. Consequently, the use of particular enological practices or specifically selected wine yeasts able to adsorb OTA during alcoholic fermentation was proposed to counter this problem (Caridi et al. 2006, 2012; Cecchini et al. 2006; Gambuti et al. 2005; Meca, Blaiotta, and Ritieni 2010; Olivares-Marín et al. 2009; Petruzzi et al. 2015). Literature data support our approach to perform a specific selection of wine yeasts able to selectively remove OTA, while protecting both phenolics and color in red wines (Aponte and Blaiotta 2016; Petruzzi et al. 2014).

Over years of isolation and clonal selection of newly isolated strains and their descendants in single spore cultures, some strains with interesting characteristics were identified. The next step consists of construction of intraspecific hybrids, starting from the already selected strains, to control whether the required characteristics may be enhanced through such hybrids.

The aim of this research was to acquire new strains of *S. cerevisiae* exhibiting opposite characteristics in terms of cell wall adsorption: very high adsorption activity toward OTA and at the same time very low adsorption activity toward the pigmented phenolic compounds contained in the musts from black grapes. Therefore, *S. cerevisiae* strains were firstly screened for main enological traits; then, the best strains were further tested for their phenolic and OTA adsorption ability. Lastly, hybrids obtained from the best strains were tested in winemaking at wineries (Fig. 1).

2. Material and methods

2.1. Pre-selection trials

The starting point were 313 strains of *S. cerevisiae* supplied by the research groups of the University of Modena and Reggio Emilia (UniMORE), University

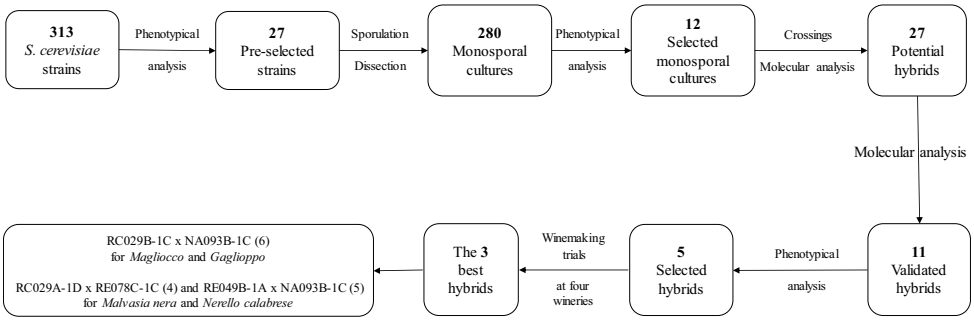

Figure 1. Experimental design.

of Napoli (UniNA), and University of Reggio Calabria (UniRC). The strains were pre-selected by evaluating: (a) type of growth designed to exclude flocculent strains during grape must fermentation in test tubes containing 10 mL of thermally treated (110°C for 10 min) and filtered (through sterile gauze) grape must, according to Caridi, Cufari, and Ramondino (2002); (b) acetic acid production to exclude high acetic acid producer strains on Chalk agar at 30°C for three days according to Lemaresquier et al. (1995); (c) H_2S production to exclude high H_2S producer strains on BiGGY agar at 25°C for two days according to Nickerson (1953); (d) production of spores typical of the genus *Saccharomyces* – to exclude non-spore and non-typical spore producer strains on acetate agar (anhydrous sodium acetate 10 g/L, agar 20 g/L) at 25°C for 10 days according to Fowell (1952).

Based on the results obtained by the pre-selection trials, the yeast strains were tested for their low adsorption activity of phenolics and high adsorption activity of OTA. To study their ability to adsorb grape pigments, the yeast strains were grown in the chromogenic grape-skin agar medium (Caridi 2013) and, after 10 days of anaerobic incubation at 28°C, yeast biomass was photographed and the images were processed for red, green, and blue components using Photoshop CS for Windows XP from Adobe. Moreover, the strains were tested in micro-winemaking trials to confirm their low ability to adsorb grape pigments and phenolics during fermentation. Black grapes of *Gaglioppo* cultivar were given pre-fermentative maceration to extract pigments from skins and seeds. They were destemmed, crushed and cold soaked at 0°C for three days, performing a punch down twice per day. The must obtained after pressing (pH 3.50, °Brix 23) was divided in aliquots of 20 mL, immediately inoculated at 5% in triplicate with the wine yeasts, and incubated at 20°C. The weight loss caused by CO_2 production after three days of fermentation was determined according to Caridi (2003); so, the fermentation vigor was expressed as g of CO_2 100 mL^{-1} of must. At the end of fermentation, wines were diluted 1:5 (v/v) with a pH 3.5 buffer (citric acid monohydrate 0.1 M, Na_2HPO_4 0.2 M) after centrifugation. The absorbance at 420, 520, and 620 nm was

read using an Anadeo1 spectrophotometer (Bibby Sterilin Ltd); the color intensity was calculated with the following formula: I = A420 + A520 + A620 (Glories 1984). The total phenolic content was determined using the Folin-Ciocalteu's index according to Singleton and Rossi (1965). Based on the results of strains ability to adsorb grape pigments and phenolics, several strains were excluded. The remaining strains were further studied for their ability to remove OTA from synthetic must (Yeast Nitrogen Base 6.7 g/L, tartaric acid 5.0 g/L, malic acid 5.0 g/L, citric acid 0.2 g/L, dextrose 110 g/L, fructose 100 g/L, and sucrose 7 g/L, pH 3.3) supplemented with 5 ppb of OTA, considering the percentage of OTA removed. Yeast pre-cultures were prepared in YPD broth (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) at 28°C for 48 h. Tests were performed inoculating in triplicate 10 mL of the synthetic must with 0.2 mL of the pre-cultures. The fermentations were carried out at 25°C and after 28 days the natural OTA content of the wines was determined by HPLC, expressing data in ppb (Meca, Blaiotta, and Ritieni 2010).

2.2. Sporulation and spore clone selection

Parents and progenies were studied using the chromogenic grape-skin agar medium and performing micro-winemaking trials to confirm their low ability to adsorb grape pigments and phenolics during fermentation. The yeasts were grown at 28°C for 2 days on YPD broth, solidified with 2% agar when required. Sporulation was induced at 28°C for seven days on acetate agar. Ascospores were isolated on YPD agar by a micromanipulator Singer MSM System series 300 manual. Ascus wall was digested at 25°C for 20 min using zymolyase 20 T – 10 mg/mL (Seikagaku, Kogyo / Tokyo, Japan) diluted 1:9 with sterile distilled water. To make sure only pure cultures were used, cells from a colony (monosporal cultures and potential hybrids) of about 1 mm diameter were picked up and suspended in 25 µL of sterile water. The cell suspension was purified through isolation by micromanipulator of only one cell, to be sure of obtaining a pure culture.

PCR reaction was performed directly on the colony by heat treatment (Ciani et al. 2003) without extracting DNA and applying the PCR conditions described by Mannazzu et al. (2002) and Mariangeli et al. (2004). The PCR amplicons were analyzed by electrophoresis on a 1.4% agarose gel in 0.5 X TBE buffer stained with ethidium bromide. The restriction fragments length polymorphism (RFLP) analysis of the SED1 and AGA1 was performed with two restriction enzymes: *Hpa* II (BioLabs, New England) for SED1 and *Alu* I (BioLabs, New England) for AGA1. The reaction mix was incubated for 2 h and analyzed on 2% agarose gel. Customized oligonucleotides were used to amplify some regions of the yeast genome between the elements that provide an amplified sequence polymorphism, useful in differentiating *S. cerevisiae* at the strain level (Legras and Karst 2003; Ness et al. 1993). Amplification

reactions were performed on a GeneAMP PCR System 2004 thermal cycler (Applied Biosystems, Foster City, California), using primers d12 and d21 and using the same experimental conditions set by Legras and Karst (2003). The amplification products were analyzed by electrophoresis on 1.8% agarose gel in 1X TBE buffer and visualized by UV light after ethidium bromide staining.

2.3. Hybrids production and molecular characterization

Hybrids were obtained according to Boveri, Raineri, and Pulvirenti (2012) and typed by molecular methods including interdelta analysis and minisatellite markers (DAN4, AGA1, SED1, HSP150) according to Boveri, Raineri, and Pulvirenti (2012) and Aponte and Blaiotta (2016).

2.4. Winemaking trials

The control strain Zymaflore F15 (Laffort Oenologie, France) and the five best hybrid strains were used in winemaking at four Calabrian wineries: 1) Azienda Agrituristica Contessa, Lattarico (CS); 2) Azienda Vinicola Malaspina, Melito Porto Salvo (RC); 3) Azienda Agricola Cosimo Murace, Bivongi (RC); 4) Azienda Agricola Fratelli Zagarella, Arghillà (RC). Therefore, grape musts of the following cultivars: *Gaglioppo, Magliocco, Malvasia nera*, and *Nerello calabrese* were used.

2.5. Analyses of the wines

The wines produced were analyzed for the absorbance at 420, 520, and 620 nm, indicating the yellow, red, and blue color, respectively and the color intensity, the total phenolic and the OTA content using the above reported methods. In addition, the experimental wines were analyzed by HPLC on a Gilson 307 Series HPLC system equipped with a refractive index detector (RID 133, Gilson) and using an MetCarb68H column (6.5 300 mm, Varian) as reported by Aponte and Blaiotta (2016). The flow rate was 0.4 mL/min and the mobile phase was 0.01 N H_2SO_4. The injection volume of mixed standards was 20 mL. The temperature of the column was set at 65°C. The identification of acetic, citric, tartaric, malic, and succinic acids and of glycerol and ethanol was carried out by comparing retention times with those of standards (wine analysis stock solutions I, II and IV, Fluka) under the same HPLC conditions. Quantitative determination was performed using the external standard method.

2.6. Statistical analysis

Data had two replicates which were subjected to statistical analysis using StatGraphics Centurion XVI for Windows XP (StatPoint Technologies, Inc., USA) according to Fisher's LSD (Least Significant Difference) ($p < .05$).

3. Results

3.1. Pre-selection trials

To increase the biodiversity of the yeast strains from which to start the research, we have found the candidates for the final selection by performing different screenings of many wine yeast strains isolated from different territories of the Mediterranean basin.

A first group of candidates was provided by the UniMORE research group. Ten strains were obtained by the screening of 111 yeast strains isolated from: a) grapes of the *Carricante, Grecanico*, and *Nerello mascalese*, cultivars, grown in the Sicily region (Italy), b) grapes of the *Grecanico, Tempranillo* and *Touriga national* cultivars, grown in the Penedès and La Rioja regions (Spain), c) grapes of the *Tinta rorizi, Touriga franca*, and *Touriga national* cultivars grown in Portugal (data not shown).

A second group of candidates was provided by the UniNA research group. Ten strains were obtained after screening of 118 yeast strains isolated from grapes of the *Catalenesca del Vesuvio, Gragnano, Moscato di Saracena*, and *Magliocco Canino* (Pollino DOC area) cultivars grown in the Campania (Italy) and Calabria (Italy) regions (data not shown).

A third group of candidates was provided by the UniRC research group. Seven strains were obtained after screening of 84 yeast strains isolated from grapes of the *Greco bianco, Inzolia, Magliocco, Nerello calabrese*, and *Pecorello* cultivars grown in the Calabria (Italy) and Sicily (Italy) regions (data not shown).

3.2. Sporulation and spore clone selection

Through dissection with Singer micromanipulator and after controlling the viability of the spores, 280 monosporal cultures were obtained from the pre-selected 27 strains. This included 113 descendants from the 10 candidates of UniMORE, 99 descendants from the 10 candidates of UniNA, and 68 descendants from the seven candidates of UniRC. In order to highlight the segregation of the desired traits, the monosporal cultures obtained were subjected to the same screening tests performed on the parental strains. As result of this screening, four monosporal cultures for each site were chosen for classic genetic improvement by using the hybridization technique (data not shown).

3.3. Hybrids production and molecular characterization

The hybridization technique is understood as a genetic improvement technique. Consequently, it is necessary to verify the variations of the phenotypic characteristics of interest of the hybrid. So the molecular investigations give

us confirmation of the crossing, while the phenotypic ones give us confirmation of the improvement or, on the contrary, of the possible loss of the traits of interest. Ultimately, the hybrid can be better or worse than the parents. Consequently, the term "genetic improvement" was adopted since the hybridization done with spores may effectively improve the yeasts for the studied traits. Obviously, the technique used is a classic genetic improvement technique.

The crossings made obtained a total of 27 hybrid strains. However, only 11 hybrids could be validated by analyzing them and the corresponding parents by molecular markers -RFLP analysis of the SED1 and AGA1 minisatellites (Boveri, Raineri, and Pulvirenti 2012).

In order to better characterize the 11 hybrids, additional molecular markers were analyzed (Table 1). The results of the genotypic analysis by different molecular markers showed that the numerous hybridization attempts led to the generation of 11 different hybrids; two hybrids – RC029A-1D x RC039C-1 C (4) and RC029B-1 C x RC039C-1 C (7) – coming from the same parents showed identical molecular markers.

The 11 hybrid strains obtained were subjected to phenotypical analysis (Table 2) to choose the best five hybrid strains, which were used to perform winemaking at the four wineries. The hybrids exhibited similar acetic acid production on Petri plates but at the end of the winemaking one of them, strain RC029A-1D x RC039C-1 C (4), exhibited a too high value (0.452 g/L of acetic acid) and was excluded. Eight out the 11 hybrids produced a low or medium content of sulfur compounds; so three strains;NA014C-1D x RC039C-1 C (3), RC026C-1 C x RC039C-1 C (9), and RE049B-1A x RC039C-1 C (9) were excluded due to their too high sulfur compounds production. The OTA removal ranged from 7.24 to 58.50%; so, two strains, NA015A-1B x NA093B-1 C (2) and NA015A-1B x RC039C-1 C (5) that exhibited the lowest adsorption of the OTA were excluded.

Table 1. Genotypic analysis of 11 verified hybrids of *Saccharomyces cerevisiae*.

	Pattern showed by different molecular markers						
Hybrid[1]	Interdelta	DAN4	AGA1	SED1	HSP150	DAN4/Rsa I	Biotype[2]
NA014C-1D x RC039C-1 C (3)	A	A	A	A	A	A	B1
NA015A-1B x NA093B-1 C (2)	B	B	B	B	A	B	B2
NA015A-1B x RC039C-1 C (5)	B	C	A	A	A	C	B3
RC026C-1 C x RC039C-1 C (9)	C	D	A	A	B	D	B4
RC029A-1D x RC039C-1 C (4)	D	E	A	C	A	E	B5
RC029A-1D x RE078C-1 C (4)	E	F	B	C	B	F	B6
RC029B-1 C x NA093B-1 C (6)	D	G	B	D	A	G	B7
RC029B-1 C x RC039C-1 C (7)	D	E	A	C	A	E	B5
RC029B-1 C x RE078C-1 C (4)	E1	H	B	C	B	H	B8
RE049B-1A x NA093B-1 C (5)	F	I	B	B	B	I	B9
RE049B-1A x RC039C-1 C (9)	F	E	A	A	B	E1	B10

[1]The five selected hybrids are in bold. [2]Biotype: on the basis of combined results of different molecular markers.

Table 2. Phenotypical analysis of 11 verified hybrids of *Saccharomyces cerevisiae*.

Hybrid[1]	Acetic acid production on Chalk agar[2]	H_2S production on BiGGY agar[3]	Production of spores on acetate agar	Fermentation vigor after 4 days (g of CO_2 /100 mL of grape must)[4]	Acetic acid (g/L)[4]	OTA removed (%)[4]
NA014C-1D x RC039C-1 C (3)	0.4	5	+	9.60 ± 0.05^{e}	0.253 ± 0.006^{d}	57.03 ± 8.11^{a}
NA015A-1B x NA093B-1 C (2)	0.3	3	+	9.82 ± 0.03^{bc}	0.255 ± 0.006^{d}	7.24 ± 2.30 ^{c}
NA015A-1B x RC039C-1 C (5)	0.4	2	-	10.03 ± 0.04^{a}	0.029 ± 0.001 ^{f}	26.05 ± 9.62^{b}
RC026C-1 C x RC039C-1 C (9)	0.3	5	+	9.77 ± 0.07 ^{cd}	0.344 ± 0.000 ^{c}	57.53 ± 6.02^{a}
RC029A-1D x RC039C-1 C (4)	0.4	3	+	9.63 ± 0.03^{de}	0.452 ± 0.006^{a}	48.07 ± 4.66^{a}
RC029A-1D x RE078C-1 C (4)	0.3	3	+	9.06 ± 0.06 ^{h}	0.395 ± 0.003^{b}	46.38 ± 2.46^{a}
RC029B-1 C x NA093B-1 C (6)	0.3	4	+	9.43 ± 0.08 ^{f}	0.319 ± 0.020 ^{c}	47.07 ± 4.27^{a}
RC029B-1 C x RC039C-1 C (7)	0.4	4	+	9.64 ± 0.04^{de}	0.273 ± 0.005^{d}	48.47 ± 4.46^{a}
RC029B-1 C x RE078C-1 C (4)	0.3	3	-	9.26 ± 0.04 ^{g}	0.026 ± 0.005 ^{f}	53.36 ± 0.96^{a}
RE049B-1A x NA093B-1 C (5)	0.3	4	+	9.90 ± 0.05^{abc}	0.074 ± 0.020^{e}	50.44 ± 9.99^{a}
RE049B-1A x RC039C-1 C (9)	0.4	5	+	9.96 ± 0.03^{ab}	0.026 ± 0.006 ^{f}	58.50 ± 3.25^{a}

Values followed by different small letters in the same column are significantly different ($p < 0.05$). [1]The five selected hybrids are in bold; [2]diameter of the clear halo around biomass (in mm); [3]color of the biomass: 1) snow; 2) white; 3) hazelnut; 4) brown; 5) rust; 6) coffee; [4]in synthetic must supplemented with 5 ppb of OTA.

3.4. Analyses of the wines

Table 3 reports the analytical traits of the wines produced at the winery Contessa using black grapes of the cultivar *Magliocco*. Compared to the control wine, produced using the wine yeast Zymaflore F15, all the five hybrids produced wines with lower content of OTA. Strain RC029B-1 C x NA093B-1 C (6) seems to be preferable since it produced wine with higher contents in ethanol and tartaric acid and with the lowest content of acetic acid.

Table 4 reports the analytical traits of the wines produced at the winery Zagarella using black grapes of the cultivar *Malvasia nera*. Compared to the control wine, all the five hybrids produced wines with significantly lower content of OTA and significantly higher content of polyphenolic compounds (Folin-Ciocalteu's index). Strain RC029A-1D x RE078C-1 C (4) seems to be preferable due to the higher contents of ethanol, tartaric, malic, and succinic acids, the significantly highest absorbance at 520 nm and color intensity, and with the lowest content of acetic acid.

Table 5 reports the analytical traits of the wines produced at the winery Malaspina using black grapes of the cultivar *Gaglioppo*. Some analyses of the wine produced with the hybrid RC029A-1D x RE078C-1 C (4) are missing because they were not detected (see "nd" in the table) andthis has no consequence on the conclusions drawn. Compared to the control wine, strain RE049B-1A x NA093B-1 C (5) seems to be preferable due to the higher content of ethanol, higher absorbance at 520 nm and color intensity, higher content of polyphenolic compounds (Folin-Ciocalteu's index), and the lowest content in OTA.

Table 6 reports the analytical traits of the wines produced at the winery Murace using black grapes of the cultivar *Nerello calabrese*. Some analyses of the wines produced with the hybrids RC029A-1D x RE078C-1 C (4) and RC029B-1 C x RC039C-1 C (7) are missing because they were not detected (see "nd" in the table) and in addition, for the same reason, the acetic acid content is missing for all the wines. This has no consequence on the conclusions drawn. Compared to the control wine, strain RC029B-1 C x RE078C-1 C (4) seems to be preferable due to the highest content of ethanol and tartaric acid, the significantly highest absorbance at 520 nm and color intensity, higher content of polyphenolic compounds (Folin-Ciocalteu's index), and to the lowest content of OTA.

It is interesting to note that in the different winemaking trials the hybrids performed differently; therefore, the importance of a correct and specific strain selection is validated.

4. Discussion

The wine industry is constantly searching for yeast strains that could result in the production of wine with better sensory and color properties. However,

Table 3. Analytical traits of the wines produced at the winery Contessa – cultivar *Magliocco*.

Strain	Ethanol (vol. %)	Acetic acid (g/L)	Absorbance 520 nm	Color intensity	Folin-Ciocalteu index	OTA (ppb)	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Glycerol (g/L)
Zymaflore F15	14.08 ± 0.11^{a}	0.374 ± 0.012^{ab}	1.920 ± 0.006 ^{f}	4.554 ± 0.020 ^{f}	59.20 ± 0.28^{d}	0.43 ± 0.05^{d}	0.710 ± 0.103 ^{c}	2.855 ± 0.402^{ab}	4.132 ± 0.885^{a}	1.796 ± 0.140^{a}	7.778 ± 0.848 ^{c}
RC029A-1D x RE078C-1 C (4)	15.35 ± 0.13^{bc}	0.579 ± 0.045^{d}	1.478 ± 0.025^{a}	3.548 ± 0.051^{a}	56.10 ± 1.27^{b}	0.12 ± 0.02^{a}	0.535 ± 0.117^{b}	2.972 ± 0.001^{ab}	4.306 ± 0.098^{a}	1.880 ± 0.037^{a}	6.730 ± 0.055^{a}
RC029B-1 C x NA093B-1 C (6)	15.27 ± 0.04^{b}	0.339 ± 0.058^{a}	1.706 ± 0.014^{d}	4.042 ± 0.042^{d}	58.00 ± 0.28 ^{c}	0.35 ± 0.06^{bc}	0.503 ± 0.097^{ab}	3.065 ± 0.134^{b}	3.963 ± 0.059^{a}	1.678 ± 0.252^{a}	7.003 ± 0.049^{ab}
RC029B-1 C x RC039C-1 C (7)	13.98 ± 0.19^{a}	0.409 ± 0.089^{bc}	1.510 ± 0.014^{b}	3.616 ± 0.051^{b}	52.20 ± 0.28^{a}	0.14 ± 0.05^{a}	0.380 ± 0.092^{a}	2.550 ± 0.503^{a}	3.634 ± 0.805^{a}	1.678 ± 0.252^{a}	6.482 ± 0.737^{a}
RC029B-1 C x RE078C-1 C (4)	13.98 ± 0.17^{a}	0.404 ± 0.009^{abc}	1.660 ± 0.017 ^{c}	3.954 ± 0.042 ^{c}	58.70 ± 0.42 ^{cd}	0.38 ± 0.05 ^{cd}	0.499 ± 0.141^{ab}	2.909 ± 0.555^{ab}	3.986 ± 0.913^{a}	1.612 ± 0.569^{a}	6.586 ± 0.719^{a}
RE049B-1A x NA093B-1 C (5)	15.49 ± 0.17 ^{c}	0.467 ± 0.067 ^{c}	1.818 ± 0.003^{e}	4.284 ± 0.000^{e}	55.90 ± 0.14^{b}	0.30 ± 0.05^{b}	0.468 ± 0.110^{ab}	2.884 ± 0.111^{ab}	3.979 ± 0.051^{a}	1.796 ± 0.141^{a}	7.502 ± 0.236^{bc}

Values followed by different small letters in the same column are significantly different ($p < 0.05$).

Table 4. Analytical traits of the wines produced at the winery Zagarella – cultivar *Malvasia nera*.

Strain	Ethanol (vol. %)	Acetic acid (g/L)	Absorbance 520 nm	Color intensity	Folin-Ciocalteu index	OTA (ppb)	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Glycerol (g/L)
Zymaflore F15	13.40 ± 0.17^{ab}	0.665 ± 0.059^{a}	1.774 ± 0.003^{a}	3.912 ± 0.017^{b}	21.77 ± 0.05^{a}	0.36 ± 0.06 ^{c}	0.278 ± 0.029 ^{c}	4.250 ± 0.563^{ab}	3.251 ± 1.005^{a}	1.139 ± 0.136^{a}	10.309 ± 0.164^{e}
RC029A-1D x RE078C-1 C (4)	13.69 ± 0.24 ^{c}	0.650 ± 0.003^{a}	1.968 ± 0.006 ^{c}	4.230 ± 0.014^{d}	23.97 ± 0.14 ^{c}	0.25 ± 0.05^{b}	0.248 ± 0.004^{b}	4.347 ± 0.111^{b}	3.698 ± 0.038^{ab}	1.333 ± 0.013^{ab}	9.105 ± 0.120^{ab}
RC029B-1 C x NA093B-1 C (6)	13.63 ± 0.17^{bc}	0.683 ± 0.029^{a}	1.858 ± 0.014^{b}	3.992 ± 0.023 ^{c}	22.93 ± 0.00^{b}	0.18 ± 0.04^{a}	0.155 ± 0.006^{a}	4.249 ± 0.228^{ab}	3.640 ± 0.177^{ab}	1.451 ± 0.082^{bc}	9.248 ± 0.104^{bc}
RC029B-1 C x RC039C-1 C (7)	13.70 ± 0.17 ^{c}	0.739 ± 0.011^{b}	1.866 ± 0.014^{b}	4.020 ± 0.023 ^{c}	25.70 ± 0.24^{d}	0.20 ± 0.05^{ab}	0.150 ± 0.000^{a}	4.050 ± 0.042^{ab}	3.586 ± 0.123^{ab}	1.680 ± 0.437 ^{c}	9.023 ± 0.127^{a}
RC029B-1 C x RE078C-1 C (4)	13.61 ± 0.17^{bc}	0.681 ± 0.045^{a}	1.724 ± 0.124^{a}	3.850 ± 0.088^{a}	25.70 ± 0.14^{d}	0.16 ± 0.05^{a}	0.271 ± 0.030 ^{c}	4.295 ± 0.559^{ab}	4.575 ± 0.796 ^{c}	1.688 ± 0.208 ^{c}	9.275 ± 0.178 ^{c}
RE049B-1A x NA093B-1 C (5)	13.35 ± 0.17^{a}	0.755 ± 0.028^{b}	1.776 ± 0.006^{a}	3.860 ± 0.023^{a}	27.57 ± 0.05^{e}	0.20 ± 0.04^{ab}	0.150 ± 0.000^{a}	3.897 ± 0.235^{a}	3.958 ± 0.251^{bc}	1.085 ± 0.207^{a}	9.711 ± 0.077^{d}

Values followed by different small letters in the same column are significantly different ($p < 0.05$).

Table 5. Analytical traits of the wines produced at the winery Malaspina – cultivar *Gaglioppo*.

Strain	Ethanol (vol. %)	Acetic acid (g/L)	Absorbance 520 nm	Color intensity	Folin-Ciocalteu index	OTA (ppb)	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Glycerol (g/L)
Zymaflore F15	12.85 ± 0.17^{ab}	0.118 ± 0.030^{a}	2.072 ± 0.000 ^{c}	4.864 ± 0.006 ^{c}	47.50 ± 0.71^{b}	0.48 ± 0.07^{d}	0.353 ± 0.074^{a}	5.426 ± 0.378^{ab}	2.894 ± 0.386^{bc}	1.779 ± 0.269^{b}	6.975 ± 0.157^{d}
RC029A-1D x RE078C-1 C (4)	12.86 ± 0.17^{b}	nd	2.582 ± 0.008^{e}	6.074 ± 0.037 ^{f}	58.10 ± 0.42^{e}	nd	nd	nd	nd	nd	nd
RC029B-1 C x NA093B-1 C (6)	12.99 ± 0.17^{b}	0.429 ± 0.047^{d}	1.824 ± 0.006^{b}	4.342 ± 0.037^{b}	46.60 ± 0.28^{a}	0.39 ± 0.05 ^{c}	0.294 ± 0.001^{a}	5.443 ± 0.130^{ab}	2.507 ± 0.114^{ab}	1.626 ± 0.030^{ab}	6.128 ± 0.026^{bc}
RC029B-1 C x RC039C-1 C (7)	12.62 ± 0.16^{a}	0.179 ± 0.025^{b}	2.230 ± 0.008^{d}	5.116 ± 0.028^{d}	55.80 ± 0.57^{d}	0.22 ± 0.06^{b}	0.272 ± 0.124^{a}	5.178 ± 0.467^{a}	2.872 ± 0.497^{bc}	1.480 ± 0.288^{a}	5.741 ± 0.368^{a}
RC029B-1 C x RE078C-1 C (4)	13.30 ± 0.17 ^{c}	0.251 ± 0.004 ^{c}	1.700 ± 0.006^{a}	4.022 ± 0.031^{a}	46.90 ± 0.14^{ab}	0.14 ± 0.05^{a}	0.341 ± 0.003^{a}	5.586 ± 0.057^{b}	3.185 ± 0.023 ^{c}	1.516 ± 0.172^{ab}	5.854 ± 0.273^{ab}
RE049B-1A x NA093B-1 C (5)	13.07 ± 0.17^{b}	0.183 ± 0.025^{b}	2.226 ± 0.014^{d}	5.190 ± 0.048^{e}	52.10 ± 0.71 ^{c}	0.13 ± 0.03^{a}	0.302 ± 0.044^{a}	5.157 ± 0.240^{a}	2.290 ± 0.357^{a}	1.497 ± 0.156^{a}	6.430 ± 0.228 ^{c}

Values followed by different small letters in the same column are significantly different ($p < 0.05$). For the wine produced using the strain RC029A-1D x RE078C-1 C (4) some data are missing because they were not detected (nd) on the analyses.

Table 6. Analytical traits of the wines produced at the winery Murace – cultivar *Nerello calabrese*.

Strain	Ethanol (vol. %)	Absorbance 520 nm	Color intensity	Folin-Ciocalteu index	OTA (ppb)	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Glycerol (g/L)
Zymaflore F15	12.56 ± 0.17^{a}	4.668 ± 0.006 ^{c}	9.704 ± 0.011 ^{c}	56.10 ± 0.99^{b}	0.18 ± 0.02^{a}	0.510 ± 0.098^{a}	4.294 ± 0.247^{ab}	4.704 ± 0.260 ^{c}	2.039 ± 0.226^{b}	7.242 ± 0.143 ^{c}
RC029A-1D x RE078C-1 C (4)	12.72 ± 0.17^{a}	3.922 ± 0.008^{a}	8.310 ± 0.003^{a}	49.00 ± 0.00^{a}	nd	nd	nd	nd	nd	nd
RC029B-1 C x NA093B-1 C (6)	12.68 ± 0.17^{a}	4.428 ± 0.000^{b}	9.292 ± 0.017^{b}	60.70 ± 1.27 ^{c}	0.18 ± 0.03^{a}	0.506 ± 0.049^{a}	4.648 ± 0.323^{b}	4.216 ± 0.571^{b}	1.529 ± 0.134^{a}	6.210 ± 0.160^{a}
RC029B-1 C x RC039C-1 C (7)	12.61 ± 0.17^{a}	4.730 ± 0.025^{d}	9.864 ± 0.051^{d}	60.50 ± 0.42 ^{c}	nd	nd	nd	nd	nd	nd
RC029B-1 C x RE078C-1 C (4)	12.76 ± 0.17^{a}	5.010 ± 0.020^{e}	10.302 ± 0.054^{e}	60.00 ± 0.85 ^{c}	0.14 ± 0.05^{a}	0.444 ± 0.001^{a}	5.461 ± 0.097 ^{c}	3.622 ± 0.077^{a}	1.642 ± 0.060^{a}	6.114 ± 0.066^{a}
RE049B-1A x NA093B-1 C (5)	12.66 ± 0.17^{a}	4.670 ± 0.048 ^{c}	9.704 ± 0.096 ^{c}	56.50 ± 0.71^{b}	0.19 ± 0.06^{a}	0.532 ± 0.103^{a}	4.177 ± 0.363^{a}	4.985 ± 0.070 ^{c}	1.668 ± 0.267^{a}	6.792 ± 0.013^{b}

Values followed by different small letters in the same column are significantly different ($p < 0.05$). For the wines produced using the strains RC029A-1D x RE078C-1 C (4) and RC029B-1 C x RC039C-1 C (7) some data are missing because they were not detected (nd) on the analyses.

when selecting yeasts, it is suggested to take into account the possible enhancement of a specific wine characteristic which could have a detrimental effect on the other wine properties (Topić Božič et al. 2019). Sidari et al. (2007) demonstrated that yeast starter can induce differences in wine color. In some circumstances, this is due to the wine color adsorption phenotype, an inheritable quantitative trait loci of wine yeasts (Caridi et al. 2007). Monagas, Gómez-Cordovés, and Bartolomé (2007) conducted a study on the influence of *S. cerevisiae* yeast strains on the anthocyanin, pyranoanthocyanins and non-anthocyanin phenolic compounds of red wines where the results showed that anthocyanins were the compounds most affected by the yeast strains, independent of the grape variety.

The main yeast selection criteria to improve wine color include: 1) the ability to enhance wine color via the metabolic formation of stable pigments, e.g., vitisins and vinylphenolic pyranoanthocyanins, and the scant adsorption of anthocyanins by the yeast cell wall; 2) the absence of β-glucosidase activity, to prevent color degradation; 3) the facilitation of colloidal stabilization in red wines by allowing aging, to help stabilize color (Suárez-Lepe and Morata 2012). Meca, Blaiotta, and Ritieni (2010) showed that yeast adsorbed the mycotoxin on the external and internal part of the cell. Moreover, OTA is mainly adsorbed during yeast exponential grow phase and in some cases, depending on strain, again released in wine (Aponte and Blaiotta 2016). This phenomenon could be due to the premature autolysis of some yeast strains.

Yeast cells adsorption activity is one of the proposed mechanisms to remove both colored phenols and OTA (Bejaoui et al. 2004; Moruno et al. 2005). Other authors have proposed OTA degradation pathway (Angioni et al. 2007). We propose, for the first time, to control red winemaking using selected hybrid yeast strains in order to remove OTA and not remove colored phenols. The results are of interest as OTA content in wines has a legal limit, at least in Europe and the most common corrective techniques to reduce it are agents like active carbon or microbial free filtration, which are efficient but have important undesirable side effects, such losses of color and aroma. Frequently, prior to bottling, the wine is treated by filtration to remove microorganisms, where which it passes through a battery of filters and goes directly to the warehouses of the cellar; this way up to 80% of OTA is usually removed. The five hybrids were chosen considering not only the OTA adsorption parameter (47–53% in synthetic must supplemented with 5 ppb of OTA) but taking also into account and balancing the other screening parameters, for example color intensity. All the wines produced using the five hybrids always exhibited no detectable OTA or OTA content under the legal limit. One treatment (hybrid yeast) did not exclude the other (filtration of microorganisms) which takes place during fermentation and the other during stabilization.

The most delicate work was to develop a certain system to confirm that the strains obtained after micromanipulation were truly hybrids deriving from the

two parents. Being parents of the same species, the techniques normally applied are not effective. With regard to the validation of the effective establishment of intraspecific hybrids deriving from the crossbreeding of monosporal cultures, selected on the basis of the characteristics "*very high adsorption of the OTA*" and "*very low adsorption of the colored phenolic compounds*", we have decided to use the comparison of the polymorphism of minisatellite-like regions contained in the two genes SED1 and AGA1. Both genes encode components of the cell wall structure, the first for membrane glycoproteins, the second for anchoring the α-agglutinin subunit to membrane components. The minisatellite-like regions, contained in the two genes, were studied and developed in laboratory protocols, adopting this system to highlight the differences in polymorphism in both the amplified and restriction mapping for identification and characterization at the level of strain and species of "*wild*" yeasts. It was previously verified that for the genes tested there are numerous allelic variants of the same minisatellite-like regions, found both by directly studying the wild-type karyotype, and the monosporal cultures deriving from the same sample (Boveri, Raineri, and Pulvirenti 2012). This last data is interesting for our research, as we work with monosporal crops and, therefore, suppose that there is an increase in polymorphism, which can be used to compare different profiles. We hypothesized that sexual recombination – hybridization mechanism – generates an acquisition of both amplification structures deriving from the two parental strains. The use of the polymorphism of the SED1 gene was not sufficient for all the pairs to be hybridized. So, in the same way we proceeded to study the amplification of the AGA1 gene to find differences between the missing pairs. This was possible because all the hybridization pairs have either SED1 or AGA1. Even with the AGA1 gene, the actual recombination is already denoted by comparing the amplified and, subsequently, always in the same modalities and principles of recombination, but with a different restriction enzyme (AluI), also by comparing the restriction profiles. Both the study of the polymorphism of the SED1 gene and that of AGA1 are an excellent and easy way to set up tool in the procedure and in the reading of electrophoretic profiles, for the validation of intraspecific hybrids. Given the importance of the yeast cell wall composition in the adsorption process of colored phenolic compounds (Caridi et al. 2017b; Mazauric and Salmon 2006; Morata et al. 2003; Sidari et al. 2007) and OTA (Chen et al. 2018), and given the direct implication of the SED1 and AGA1 genes in some of the membrane components, it is assumed that the choice of these genes as molecular targets for the validation of intraspecific hybrids is of interest.

The natural variability of yeasts for the studied traits may be exploited to obtain a further enhancement of the adsorption/non-adsorption activity of wine yeasts. However, the trend of the yeast adsorption performance is that strains that remove more OTA are the same that adsorb more color and phenolics from wine. Consequently, the best strains for red winemaking obtained with the present selection possess intermediary adsorption

characteristics. The inheritable nature of the adsorption of wine color and OTA were analyzed on descendants derived from wine strains of *S. cerevisiae* based on the investigation on the progeny which demonstrated that adsorption of wine color and adsorption of OTA are polygenic inheritable quantitative traits loci, partially and interdependently correlated to color and phenolic content of wines. This may justify the employment of genomic strategies for genetic improvement of the strains.

5. Conclusion

The employed experimental protocol has allowed to select yeast strains for innovative characteristics connected to their different adsorption activity, allowing to improve the strains by spore clone selection and construction of intraspecific hybrids. The results encourage to continue in this direction to enhance the traits of interest. Taking into account the results, it is possible to affirm that the choice of the most promising strain depends also on the grape cultivar used. The hybrid RC029B-1 C x NA093B-1 C (6) is the best strain in the winemaking of *Magliocco* and *Gaglioppo* cultivars while the hybrids RC029A-1D x RE078C-1 C (4) and RE049B-1A x NA093B-1 C (5) is the best strains in the winemaking of *Malvasia nera* and *Nerello calabrese* cultivars. The proposed methodology efficiently removes OTA and does not result in side effects such as loss of aroma and color, which were previously reported for other methodologies employed at industry level to deal with the problem.

Disclosure statement

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