PROTECTIVE EFFECT OF VEGETAL EXTRACTS AGAINST ACIDIC AND ALCOHOLIC STRESS IN WINE YEASTS

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Introduction

Protective agents may minimize osmotic and thermal stresses in wine yeasts (Caridi, 2002).
In the present work, the tolerance of ten strains of *Saccharomyces cerevisiae* - two wild types (RC029 and RC039) and eight descendants obtained by micromanipulation according to Caridi et al., 2017) - against three stressful agents - acetic acid, ethanol, and pH - was studied by spot dilution assay, with or without the addition of six protective vegetal extracts.

Materials and methods

Olives (cultivar *Carolea*), black grapes (cultivars *Gaglioppo* and *Magliocco canino*), and pomegranate were purchased at a local market in Reggio Calabria in October 2016.
For the extraction of phenolic compounds, 10 g of each fresh sample - olive pulps, black grapes (cultivar *Gaglioppo*), black grape skins (cultivar *Magliocco canino*), black grape seeds (cultivar *Magliocco canino*), pomegranate albedo, pomegranate arils - were ground, then vigorously vortexed for 1' with 30 mL of distilled water and the mixture was subjected to sonication for 20'. Then, 70 mL of acetone, refrigerated at -20°C, were added and the mixture was vigorously vortexed for 5'. The mixture was centrifuged at 5,000 rpm for 20' and the supernatants were collected and were concentrated in a rotary evaporator at 60°C until about 25 mL. The obtained extracts were adjusted at the final volume of 30 mL and stored at -20°C.

The total phenolic content of the six vegetal extracts was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). One hundred microliters of each extract were treated with Folin-Ciocalteu solution, incubated for 10' and analysed at 725 nm by a spectrophotometer UV–VIS. The results were reported as mg gallic acid equivalent g^{-1} of dry weight (mg GAE g^{-1}).

The total antioxidant activity determination of the six vegetal extracts is based on the reaction mechanism between the $DPPH^{\cdot}$ (2,2-diphenyl-1-picrylhydrazyl) and the antioxidants present in the samples, as reported by Brand-Williams et al. (1995). The reaction started adding 10 µL of each extract - diluted 1:10 - to 2,990 µL of a 6 x 10^{-5} M of methanol solution of $DPPH^{\cdot}$ in a cuvette, leaving it in the dark at room temperature for 15', until colour stabilisation. The decrement of absorbance was determined by a spectrophotometer at 515 nm against methanol as blank and at the temperature of 20°C to eliminate the risk of thermal degradation of the tested molecules (Bondet et al., 1997).

The Trolox Equivalent Antioxidant Capacity (TEAC) method was performed as reported by Re et al., 1999. This analysis evaluates the capacity of the sample to inhibit ABTS radical [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] oxidation, compared with a standard antioxidant (Trolox). The

reaction mixture was obtained by mixing 2,990 µL of ABTS (measuring between 0.500 and 1.000 of absorbance at 734 nm) and 10 µL of each extract, diluted 1:10. Followi

For both antioxidant assays, the results were reported as inhibition percentage. The applied formula was: **% inhibition = $[(At_0-At_e)/At_0]*100$**, where At_e is the absorbance measured after 15' (for DPPH) and 6' (for ABTS) while At_0 is the absorbance of $DPPH^{\cdot}$ and ABTS solutions at the initial time. The total antioxidant activity determined by DPPH and ABTS assays was expressed as percentage of inhibition.

The protective activity of the six vegetal extracts against the three stressful agents was measured according to the growth intensity of the yeasts on YPD agar. For each yeast strain, a suspension measuring 0.100 of absorbance at 600 nm was prepared and ten-fold diluted four times; 5 µL of each dilution were spotted on YPD agar modified by adding the stressful agents with or without the protective agents.

For the stressful agents, preliminary analyses showed that the more appropriated level of stressful agent is: a) pH 2.40, after testing pH 2.40, pH 2.60, and pH 2.80; b) ethanol 16 vol. %, after testing ethanol 8 vol. %, 10 vol. %, 12 vol. %, 14 vol. %, 16 vol. %, and 18 vol. %; c) 0.4% of acetic acid, after testing 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, and 0.8% of acetic acid.

For the protective agents, preliminary toxicity trials proved that the addition of 25 µg/mL of the phenolic extracts was not toxic for the yeast cells. Therefore, in order to obtain this final phenolic concentration for each vegetal extract, 1.125 mL of olive pulp extract, 0.921 mL of black grape extract, 0.089 mL of black grape seed extract, 1.087 mL of black grape skin extract, 0.114 mL of pomegranate albedo extract, and 0.500 mL of pomegranate aril extract were correspondingly added to 100 mL of a freshly-sterilised YPD agar.

All the chemical and microbiological experiments were conducted in triplicate.

Results and discussion

The highest total antioxidant activity was registered, respectively using DPPH and ABTS assays, for the extract of pomegranate albedo (94.60 and 60.07%) and black grape seeds (91.26 and 88.41%), followed by pomegranate arils (37.81 and 21.33%), black grapes (16.75 and 5.00%), black grape skins (9.34 and 10.55%), and olive pulps (3.19 and 5.52%). The total phenolic content was 6,670 - 8,589 - 1,518 - 868 - 756 - 664 mg GAE g^{-1}, respectively. Under ordinary pH condition (pH 6.50 ± 0.20), the four-serial dilution of yeast cells spotted on YPD agar showed the growth of all the studied ten yeast strains (Figure 1).

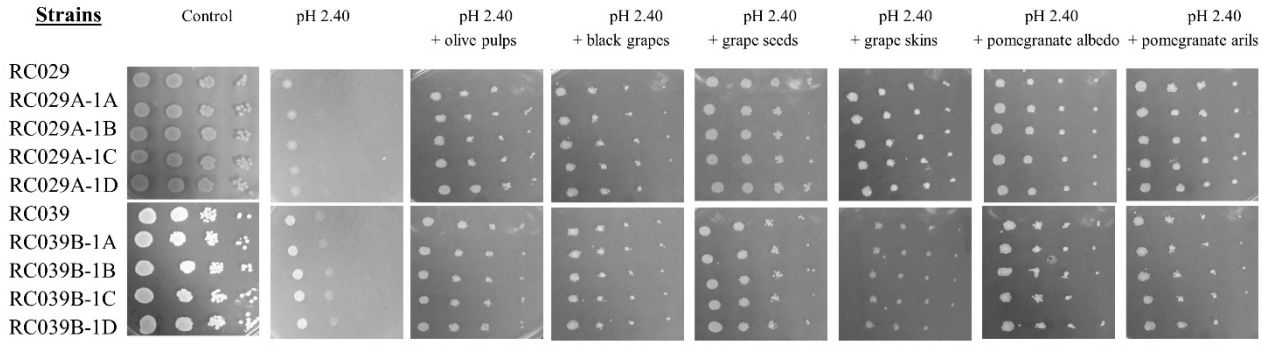

Figure 1: *Growth at four dilutions (from the left to the right, 10^0, 10^{-1}, 10^{-2} and 10^{-3}) of the ten yeast strains on YPD agar at pH 6.50 ± 0.20 (control) and pH 2.40 with or without the addition of the protective vegetal extracts.*

In contrast, under pH 2.40 conditions, and without the addition of any protectant, the yeasts did not succeed to grow when diluted more than 10^{-1}. As expected, the acidic pH stressed yeast cells and reduced their growth rate. Interestingly, the obtained results, when adding vegetal extracts to the culture media, showed that almost all the used extracts notably improved the yeast performance, allowing the growth also at the fourth (10^{-3}) dilution. Yeast cells seem to retrieve their growing ability in presence of the vegetal extracts, but always remaining at a growth rate lesser than that of the control (under ordinary pH condition). The best protective effect was obtained using the black grape seed extract. In the absence of ethanol in the growth medium, the four-serial dilution of yeast cells spotted on YPD agar showed the growth of all the studied ten yeast strains (Figure 2).

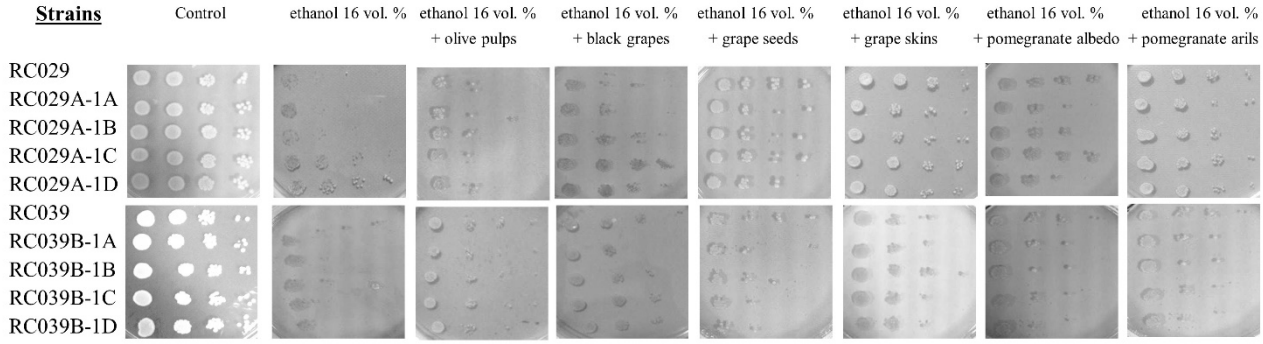

Figure 2: *Growth at four dilutions (from the left to the right, 10^0, 10^{-1}, 10^{-2} and 10^{-3}) of the ten yeast strains on YPD agar (control) and on YPD agar containing ethanol 16 vol. % with or without the addition of the protective vegetal extracts.*

In contrast, on YPD agar containing ethanol 16 vol. % without the addition of any protectant, the yeasts reduced their growth. In this case the performance is different from strain to strain. For example, strain RC029 exhibits growth until the fourth dilution when the addition of the extracts of black grape seeds, black grape skins, pomegranate albedo or pomegranate arils is performed; on the contrary, in the presence of the same protectants the strains RC039B-1C and RC039B-1D do not exhibit growth at the fourth dilution. On the whole, this time the best results are obtained using the black grape skin extract. In the absence of acetic acid in the growth medium, the four-serial dilution of yeast cells spotted on YPD agar showed the growth of all the studied ten yeast strains (Figure 3).

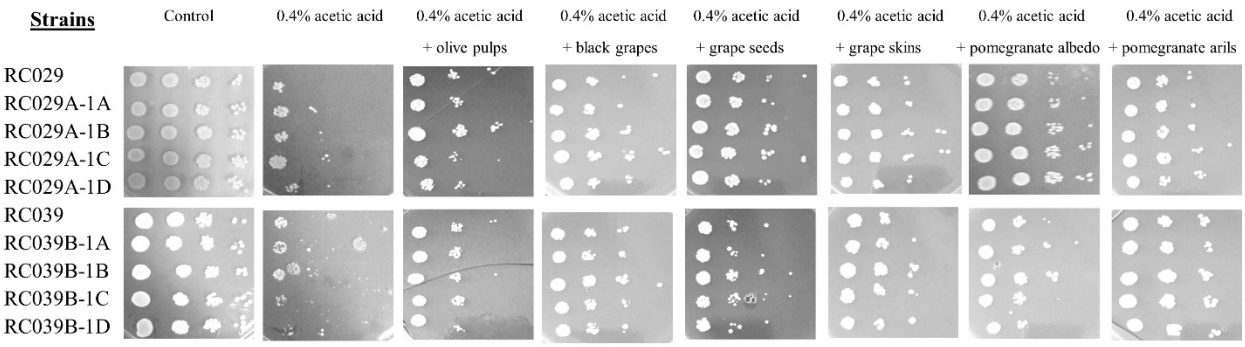

Figure 3: *Growth at four dilutions (from the left to the right, 10^0, 10^{-1}, 10^{-2} and 10^{-3}) of the ten yeast strains on YPD agar (control) and on YPD agar containing 0.4% of acetic acid with or without the addition of the protective vegetal extracts.*

In contrast, on YPD agar containing 0.4% of acetic acid without the addition of any protectant, the yeasts reduced their growth. Compared to the growth on YPD agar containing 0.4% of acetic acid without the addition of any protectant, all the vegetal extract notably improved the yeast performance. Considering the strain RC029 and their four descendants the best results are obtained using the pomegranate albedo extract; instead, considering the strain RC039 and their four descendants the best results are obtained using the pomegranate aril extract.

Conclusions

On the whole, the six vegetal extracts exhibited significantly different effects on the ten yeast strains and, in general, they allow cells to overcome the stressful agents' effects when added to the culture medium, thus, permitting to retrieve better growth rate of the yeast cells. Among the most effective protectants, some are of oenological origin. Consequently, the prolongation of the marc contact with grape must during the winemaking - in monitored conditions - could be effective to increase tolerance to ethanol- and acid-stress in wine yeasts. Considering the remarkable anti-stress activity of grape seeds, their addition to the grape must - after having powdered them - could be hypothesized to improve the fermentative performance of wine yeasts. The extract from olive pulps has shown the least anti-stress activity; consequently, its use can be excluded without the need to formulate any hypothesis for its possible use in oenology. On the contrary, the use of the two pomegranate extracts - very effective - could constitute an interesting innovation in winemaking if their anti-stress effect is not related to sensory damages to the wines, with regards to their tannin content which is known to make hazes in beverages.

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Abstract

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