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Control of *Brettanomyces bruxellensis* on wine grapes by post-harvest treatments with electrolyzed water, ozonated water and gaseous ozone

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ABSTRACT

In this study, we investigated the possible effect of electrolyzed water (EW), aqueous ozone (WO) and gaseous ozone (GO) on *Brettanomyces bruxellensis* DSM 7001 strain artificially inoculated on the grape surface and on its evolution during the subsequent, inoculated must fermentation. Culture-dependent and -independent techniques were used to evaluate the effectiveness of treatments against *B. bruxellensis*, as well as its presence during fermentation. Particularly, GO treatment of 24 h decreased its presence by about 2.1 Log, making it possible to reduce significantly the concentration of ethylphenols in the wine in relation to the control wine. EW and WO treatments caused less relevant reductions. The results showed that all the treatments reduced the presence of this yeast on grapes. However, in these experimental conditions it was not possible to achieve a complete removal of this undesirable yeast.

Industrial relevance: Brettanomyces spp. is considered a wine spoilage yeast due to its ability to produce off-flavors (described as Brett character) and high levels of acetic acid. Broad disinfectant action against microorganisms, eco-friendliness and easiness of on-site application are among the main advantages of the ozone and the electrolyzed water. This study demonstrated the antimicrobial potential of the EW, WO and GO treatments against *B. bruxellensis* inoculated on post-harvest grapes.

1. Introduction

During the alcoholic fermentation, yeasts convert sugars present in must, mainly to ethanol, but other compounds, important for the sensory characteristics of the wine, are produced as well, therefore their impact on wine quality could not be ignored (Fleet, 2008). The grape berries surface represents an important vector for yeast populations in the must. Especially when damaged berries are taken into consideration, they can carry a high number of undesirable yeast cell populations (Barata, Malfeito-Ferreira, & Loureiro, 2011; Guerzoni & Marchetti, 1987; Pretorius, 2000). Among these, *Brettanomyces bruxellensis* was isolated from several vineyards and in different stages of grape berry development, using mainly enrichment media (Renouf et al., 2006; Renouf & Lonvaud-Funel, 2007). The yeasts belonging to the genus *Dekkera/Brettanomyces* are mainly responsible for wine spoilage during its storage in cellars, particularly in red wines. These yeasts are generally known for their capacity in the wines to produce off-flavors due to the activity of two enzymes: cinnamate decarboxylase and vinyl phenol reductase (Suarez, Suarez-Lepe, Morata, & Calderon, 2007). Vinyl- and ethyl-phenols are the off-flavor compounds produced by these enzymes from hydroxycinnamic acids, which are naturally present in grape must (Benito, Palomero, Morata, Uthurry, & Suárez-Lepe, 2009). 4-Ethylphenol has a low threshold of sensory perception (350 to 1000 μg/L as a function of wine characteristics) and different flavors, like pharmaceutical, horse-like, barnyard-like, horse blanket, wet dog, tar, tobacco, creosote, leathery and perhaps mousey descriptors (Campolongo, Siegumfeldt, Aabo, Cocolin, & Arneborg, 2014; Suarez et al., 2007). In addition, *Brettanomyces* spp. is a producer under certain conditions of the "mousy" off-flavor and of high concentrations of acetic acid from the sugar metabolism (Freer, Dien, & Matsuda, 2003; Romano, Perello, De Revel, & Lonvaud-Funel, 2008; Snowdon, Bowyer, Grbin, & Bowyer, 2006). This species is considered dangerous because of its ability to survive in relatively high concentrations of ethanol (Suarez et al., 2007). Furthermore, *Brettanomyces* spp. growth control in

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wineries is very difficult due to its ability to tolerate normal concentrations of sulfur dioxide used in cellars (Cocolin, Rantsiou, Iacumin, Zironi, & Comi, 2004). Therefore, it may contaminate wineries with a low level of cleaning and disinfection. In fact, these yeasts can survive, proliferate and contaminate the wine during various steps of wine-making process. Several studies have demonstrated the risks of the presence of *Brettanomyces* spp. in wines, however it is very difficult to understand when contamination begins. As *B. bruxellensis* is frequently associated with barrel-red wines, wood used in storage and aging may be a common vector for the introduction of this species in wine in red wine (Suarez et al., 2007). However, some strains have been isolated from the vineyard (Renouf & Lonvaud-Funel, 2007). In particular, Renouf & Lonvaud-Funel (2007) where able to isolate *Brettanomyces* spp. from grape berries by using an optimized enrichment broth, able to recover their populations in a culture- dependent manner indicating that grapes may act as a possible vector for the introduction of this yeast species into the wine.

In the last years, new disinfecting agents are being proposed for fruits and vegetables treatment, such as ozone (O_3) and electrolyzed water (EW) (Boonkorn et al., 2012; Guentzel, Lam, Callan, Emmons, & Dunham, 2010; Hricova, Stephan, & Zweifel, 2008; Smilanick, Margosan, & Mlikota Gabler, 2002.). EW has a broad spectrum of action against various microorganisms thanks to three combined actions: hydrogen ions, oxidation-reduction potential and free chlorine, while, ozone is a strong oxidant able to attack several cellular constituents of the microorganisms, in addition to this, eco-friendliness and easiness of on site application are other main advantages of these agents (Jermann, Koutchma, Margas, Leadley, & Ros-Polski, 2015; Khadre, Yousef, & Kim, 2001).

On the grape, ozone is a sanitizer that leaves no residues, while a possible eventual residual of free chlorine could be a problem for the formation in vinification of chloroanisoles and chlorophenols, compounds responsible of the “cork taint” in the wines (Guentzel et al., 2010). However, to our knowledge, relationships between use of EW and presence of anisols are still not described in scientific literature. The ability of ozone and EW to sanitize has already been studied on both fresh and withered wine grapes, highlighting not only an antimicrobial effect but also an improvement of grape characteristics and wine quality (Bellincontro, Catelli, Cotarella, & Mencarelli, 2017; Paissoni et al., 2017; Río Segade et al., 2017). Considering the impact on fermentative yeasts, in grapes treated with ozone and EW, apiculate yeasts were reduced by 0.5 Log CFU/mL when compared to untreated grapes, resulting in a decrease of the acetic acid content in the wines (Cravero, Englezos, Rantsiou, et al., 2016; Cravero, Englezos, Torchio, et al., 2016).

However, studies assessing the effect of these innovative sanitizing techniques on *Brettanomyces* spp. present on the grapes are missing. Therefore, the objective of this work was to evaluate the effect of ozone (either in liquid or gaseous treatments) and EW on *B. bruxellensis* DSM 7001 on grape berries used for red wine production. Its presence in wine grapes after the treatments and during the fermentation process was studied by culture-dependent (traditional plate counts) and culture-independent (PCR-denaturing gradient gel electrophoresis [DGGE] and reverse transcription PCR [RT-PCR]-DGGE) techniques. The concentration of off-flavor compounds in the wines was determined by Head Space Solid Phase Micro-Extraction (HS-SPME) coupled to Gas Chromatography-Mass Spectrometry (GC–MS).

2. Materials and methods

2.1. Grapes and preparation of the Brettanomyces bruxellensis inoculum

Whole bunches of *Vitis vinifera* L. cultivar Barbera grapes were harvested from a vineyard located in the Asti province (Piemonte, NW Italy). They were characterized by good phytosanitary conditions, that is without signs of damage/infection by *Botrytis cinerea* or other grape pathogens, and all the skin was intact. The grapes were subdivided in small clusters of 6–8 berries. Afterwards, they were placed in a single layer into perforated boxes, forming batches of 2.0 ± 0.1 kg each. Each trial was inoculated with *B. bruxellensis* DSM 7001 strain from DSMZ, German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) (Campolongo et al., 2014). Even though the real load of *B. bruxellensis* on grapes is normally lower, in this work, we inoculated about 6.0 Log cells/mL prior to treatments, in order to accurately quantify the effects of the treatments on the yeast population. Inoculum was prepared by introducing a pure *B. bruxellensis* DSM 7001 colony into 5 mL of DBDM broth selective for *B. bruxellensis* (Campolongo, Rantsiou, Giordano, Gerbi, & Cocolin, 2010), after about 10 days incubation at 25 °C, a small aliquot of this broth was spread into DBDM agar selective medium for *B. bruxellensis*. The plates were incubated for 15 days at 25 °C, and then scraped using sterile Ringer's solution (Oxoid, Milan, Italy), thus obtaining the solution used for the inoculum. Afterwards, the yeast cells were stained with methylene blue dye and immediately the viable cell population was counted by using a Thoma hemocytometer chamber (BRAND GMBH + CO KG, Wertheim, Germany). Before inoculation, appropriate amounts of inoculum were calculated and subsequently used to inoculate the grape berry surfaces at an initial cell population of 10^8 cells/mL. Each grape aliquot was sprayed with 100 mL of inoculum. Inoculated grapes were left for 24 h at a constant temperature of about 25 °C to allow the inoculum to dry and stick to the grape skin. Grape inoculation density was verified by randomly picking thirty berries from each perforated box. Prior to inoculation, the absence of *B. bruxellensis* on grapes was checked by plate counts.

2.2. EW and ozone treatments

EW solution was generated using an EVA SYSTEM® 100 equipment (Industrie De Nora S.pA, Milano, Italy) as previously described by Cravero, Englezos, Rantsiou, et al. (2016), while an ozone generator (Model C32-AG, Industrie De Nora S.p.A, MI, Italy) was used for aqueous (WO) and gaseous (GO) ozone production (Cravero, Englezos, Torchio, et al., 2016; Laureano et al., 2016).

For the EW and WO treatments, samples were steadily sprayed for a contact time of 6 and 12 min with a nozzle connected to a peristaltic pump (SP311, Velp Scientifica, Usmate, MB, Italy). The EW solution had a concentration of 400 mg/L of free chlorine, while the WO solution had an ozone concentration of 5.00 ± 0.25 mg/L. During treatments, the flow and the temperature were maintained constant at 200 mL/min and 25 °C, respectively. Control treatments were performed using tap water.

Two different times were used for the GO treatments (12 and 24 h) in a chamber saturated with gaseous ozone at a concentration of 32 ± 1 μL/L. The treatment was performed in controlled conditions of temperature (20 ± 1 °C), relative humidity (57 ± 3%) and at constant concentration of ozone, which was constantly monitored through a UV-photometric ozone analyzer BMT 964 (BMT Messtechnik Gmbh, Germany) that controls the generator output. Control treatments were performed in another chamber for 12 and 24 h in contact with air, using the abovementioned temperature and relative humidity conditions.

For each treatment, we have used three replicates and the experimental plan is summarized as follows: **WA**: treated with tap water for 6 min (control); **WB**: treated with tap water for 12 min (control); **EWA**: treated with electrolyzed water for 6 min; **EWB**: treated with electrolyzed water for 12 min; **WOA**: treated with ozonated water for 6 min; **WOB**: treated with ozonated water for 12 min; **GA**: untreated for 12 h (control); **GB**: untreated for 24 h (control); **GOA**: treated with ozone gas for 12 h; **GOB**: treated with ozone gas for 24 h.

2.3. Laboratory-scale fermentations

For each trial, before and after treatments, about 30 berries were