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Short communication

Filamentous fungi associated with natural infection of noble rot on withered grapes

M. Lorenzini[a], B. Simonato[a], F. Favati[a], P. Bernardi[b], A. Sbarbati[b], G. Zapparoli[a,*]

[a] Dipartimento di Biotecnologie, Università degli Studi di Verona,strada Le Grazie 15, 37134 Verona, Italy
[b] Dipartimento di Neuroscienze, Biomedicina e Movimento, Università degli Studi di Verona, piazzale L.A. Scuro 10, 37134 Verona, Italy

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ABSTRACT

The effects of noble rot infection of grapes on the characteristics of different types of wine, including Italian passito wine, are well known. Nevertheless, there is still little information on filamentous fungi associated with noble-rotten grapes. In this study, withered Garganega grapes for passito wine production, naturally infected by noble rot, were analyzed and compared to sound grapes. Skin morphology and fungal population on berry surfaces were analyzed. Scanning electron microscopy analysis revealed microcracks, germination conidia and branched hyphae on noble-rotten berries. *Penicillium*, *Aureobasidium* and *Cladosporium* were the most frequent genera present. Analysis of single berries displayed higher heterogeneity of epiphytic fungi in those infected by noble-rot than in sound berries. *Penicillium adametzoides*, *Cladosporium cladospoirioides* and *Coniochaeta polymorpha* were recovered. These, to the best of our knowledge, had never been previously isolated from withered grapes and, for *C. polymorpha*, from grapevine. This study provided novel data on noble rot mycobiota and suggests that fungi that co-habit with *B. cinerea* could have an important role on grape and wine quality.

1. Introduction

The term “noble rot” indicates the endophytic infection of *Botrytis cinerea* on grape berries that occurs under specific enviromental conditions (Ribéreau-Gayon et al., 2006). The development of noble rot on grapes can occur on-vine or off-vine in the case of post-harvest storage. This latter occurrence can be observed in withered grapes, carried out in closed and ventilated rooms called *fruttaio* (fruit-drying room), used for the production of Italian passito wines (Mencarelli and Tonutti, 2013). The influence of noble rot on wine aroma of some of these wines has been analyzed (Fedrizzi et al., 2011). The positive effects of this fungus on the sensory characteristics of passito sweet wines have encouraged controlled botrytization in post-harvest conditions (Mencarelli and Tonutti, 2013; Tosi et al., 2013).

To date, natural withering is the most common grape dehydration process for passito wines production and the occurrence of noble rot infection on grapes is highly variable depending on seasonal and dehydration conditions (Mencarelli and Tonutti, 2013). During withering, winemakers remove damaged and decayed bunches or berries, which are a potential source of infection and wine defects, leaving only those that are sound or affected by noble rot. Nevertheless, in favourable conditions the activation of latent infections on these remaining berries can cause fungal disease outbreaks. In particular, the switch from noble rot to gray mould could be rapid, as could be the saprophytic colonization of berries by other fungi.

Withered grapes are colonized by several fungal species, including those that have an important pathogenic role (Lorenzini et al., 2016). Up to now however, the ecology of filamentous fungi in withered grapes has scarcely been investigated because they do not play a crucial role on grape fermentation such as yeasts. Non-*Botrytis* fungal species can affect the noble rot development and lead to disease occurrence with negative effects on grape and wine quality (Rousseaux et al., 2014). However, the fungal consortium associated with noble-rotten grapes is still largely unknown.

The present study analyzed filamentous fungi associated with infection of noble rot on Garganega grapes that frequently occurs during natural withering of this local variety that is used for the production of passito sweet wines such as Recioto and Vin Santo. Skin morphology, frequency of main fungal families or genera and species identification of isolates from single berries were performed.

2. Materials and methods

2.1. Grape sampling and berry classification

Grape samples of Garganega variety withered in natural conditions

* Corresponding author.
E-mail address: giacomo.zapparoli@univr.it (G. Zapparoli).

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with the traditional surmaturation technique, were collected in a fruit-drying room located in the Soave winemaking area (Italy) after five months from the harvest (vintage 2015). The incidence of noble-rotten grapes, estimated by visually inspection, was approximatevly 50–60%. Berries were classified in three categories (Fig. S1): "sound" when undamaged with homogeneus yellowish to amber skin, swollen or partially shrivelled; "noble-rotten" when undamaged with light to dark brown skin, partially or largely shrivelled (mummy-like); "damaged and decayed" when even partially rotted, with visible fractures or craks, partially or totally covered with mycelium. This last category was not considered in this study since generally they are discarded and not vinificated. Two individual batches of sound and noble-rotten (about 500 g each) were randomly selected from single clusters and aseptically transferred to laboratory for analysis. The presence of *B. cinerea* in noble-rotten berries and its absence in sound berries was confirmed by performing a species-specific PCR analysis using as template DNA extracted from an amount of 50 g of berries. DNA extraction was carried out according to Rezaian and Krake (1987), while PCR assay was performed using Bot-F and Bot-R primers as described by Lorenzini and Zapparoli (2014) (Fig. S2).

2.2. Scanning electron microscopy analysis of berries

Representative berries of each category (three sound and four noble-rotten), randomly selected from each batch, were analyzed by scanning electron microscopy (SEM, ESEM XL30, FEI-Philips, Hillsboro, OR) after sample preparation. Then, the samples were fixed in 2% w/v glutaraldehyde in phosphate buffer for 3 h and then dehydrated in graded acetones. Then the samples were treated by critical point dryer (CPD 030, Bal-tec, Balzers, Liechtenstein), mounted on metallic specimens stubs, sputter-coated with gold (MED 010 Balzers), and examined by SEM.

2.3. Determination of water activity of berries

Water activity (a_w) was measured in two categories of berries (sound and noble-rotten) and determination was carried out using a Hygropalm HC2-AW (Rotronic Italia srl, Milan, Italy) apparatus equipped with a thermostated stainless steel sample holder (WP-40TH, Rotronic Italia srl). Sound and noble-rotten berries, randomly selected from each batch, were placed in disposable supports and a_w determination was carried out at constant temperature of 25 °C. Measurements for each category were carried out in triplicate and the average a_w value with standard deviation was reported. The *t*-test was applied to test for statistical differences between noble-rotten and sound berries.

2.4. Isolation and identification of fungi

A total of 116 berries (49 sound and 67 infected by noble rot), randomly selected from each batch, were individually used for fungal isolation. Each berry was directly plated by rolling onto malt extract agar (MEA, 2% w/v malt extract, 0.1% w/v peptone, 2% w/v dextrose, 1.5% w/v agar) and DG18 agar (22.0% w/v glycerol, 1% w/v dextrose, 0.5% w/v peptone, 0.1% w/v potassium dihydrogen phosphate, 0.05% w/v magnesium sulfate, 0.01% w/v chloramphenicol, 0.0002% w/v dichloran, 1.5% w/v agar). After incubation for two–four days at 25 °C, individual colonies were isolated and purified through repeated streaking on MEA and DG18 agar. About 550 isolates were stored on MEA slants at 4 °C.

Classification of isolates grown on MEA, potato dextrose agar (Difco, Laboratories, Detroit, MI), oatmeal agar and yeast extract sucrose agar (Samson et al., 2004) was carried out evaluating the colony morphology and microscopic features according to Pitt and Hocking (2009) and by comparison with morphological characteristics of strains from withered grapes previoulsy identified by Lorenzini et al. (2016). All isolates were classified at family or genus level. A total of 200 isolates were identified at species level and 182 out of them (65 from sound and 117 from noble-rotten berries) were isolated from a representative berry sample. This berry sample was costituted by 15 sound and 21 noble-rotten berries (about 30% of 116 berries) selected according to the number of fungal genera and families recognized on each berry. *Botrytis cinerea*, *Epicoccum nigrum*, *Alternaria alternata* species-group and *Aureobasidium pullulans* complex were mainly recognized by deeper morphological analysis (e.g. mycelium, vegetative and/or reproductive structures, conidiophores, conidial patterns, conidia). Species attribution of these isolates was confirmed by phylogenetic analysis carried out only on representative strains. Classification at species level of *Penicillium*, *Aspergillus* section *Nigri*, *Cladosporium*, *Fusarium*, Botryosphaeriaceae and Mucoraceae, including isolates belonging to other genera, was mainly carried out by phylogenetic analysis due to high morphological similarity among species of the same genus.

2.5. DNA amplification and sequence analysis

DNA was extracted from pure culture of isolates as previously described (Lorenzini and Zapparoli, 2014). Partial gene or region sequences used for phylogenetical analysis were Internal Transcribed Spacer (ITS) using the primer pairs ITS1/ITS4 (White et al., 1990), Large Subunit (LSU) with primer pairs LR0R/LR7 (Rehner and Samuels, 1994; Vilgalys and Hester, 1990) and Actin (ACT) with primer pair ACT-512F/ACT-783R (Carbone and Kohn, 1999).

The amplification conditions were carried out as described by White et al. (1990) for ITS (ITS1/ITS4), de Gruyter et al. (2009) for LSU (LR0R/LR7), Carbone and Kohn (1999) for ACT.

The amplification products were visualized by agarose gel electrophoresis (1% w/v) and purified using the NucleoSpin gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Sequencing of these products was carried out in both directions using the same primers as for amplification (Eurofins Genomics, Ebersberg, Germany).

2.6. Phylogenetic analysis

Phylogenetic analysis was conducted using sequences from the Clustal W multiple alignment output using neighbor-joining (NJ) statistical method and maximum composite likelihood (ML) substitution model in the MEGA 7.0 interface. The phylogeny trees inferred from each sequence dataset were constructed by the NJ method and individually tested with a bootstrap (BS) of 1000 replicates to ascertain the reliability of a given branch pattern in each NJ tree.

For studying phylogenetic relationships, sequences of reference strains closely related to isolates from withered grapes object of this study were recovered from the literature (Friebes et al., 2016; Sandoval-Denis et al., 2016; Visagie et al., 2014).

3. Results and discussion

3.1. SEM and water activity analysis in berries

SEM analysis of berry skin revealed frequent presence of microcracks and wounds, conidia, germination conidia, branched hyphae and mycelium on noble-rotten berries, while mostly rare on the skin of sound berries (Fig. 1). Microcracks on skin play an imporant role in susceptibility to conidia germination and subsequent mycelial growth (Padgett and Morrison, 1990). This SEM data suggest possible effects of noble rot infection on the occurrence of microcracking although a deeper analysis (e.g., determination of frequency of microcraks on berry cuticular membrane) is recommended.

Water activity values differed significantly ($p < 0.05$) between sound and noble-rotten berries, 0.906 (± 0.004) in sound berries, 0.893 (± 0.005) in noble-rotten berries. Low water availability on