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High-throughput amplicon sequencing-based analysis of active fungal communities inhabiting grapevine after hot-water treatments reveals unexpectedly high fungal diversity

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ABSTRACT

The ecology of total fungal communities in grapevine is so far largely derived from studies on culture-dependent methods or cultivation-independent rDNA approaches. Sequencing the ribosomal RNA transcripts (rRNA) would rather reveal the functionally and metabolically active important taxa of the fungal community and provide insights into its activity in the wood. The present study investigated changes in the potentially active fungal communities of internal grapevine wood after Hot-Water Treatment (HWT) in planting material from Czech Republic and Spain at two different times during the propagation process and from two plant zones. We examined fungal communities using both traditional isolation into culture and high-throughput amplicon sequencing (HTAS) of the internal transcribed spacer 2 (ITS2) region in extracted total RNA. HTAS from metatranscriptomic RNA increased the resolution of the fungal community analysis and revealed a highly diverse mycobiota of grapevine wood compared to the traditional method. Fungal diversity differed between grapevine genotypes and showed a temporal variation over the vegetative period. Grapevine planting materials exhibited high fungal diversity after HWT, which demonstrates that the HWT process does not sterilize the internal wood of grapevine. HWT reduced the infection caused by fungal trunk disease pathogens but was not completely effective in eliminating their growth. This study provides important and practically useful insights into the dynamics of active fungal communities in hot-water treated plants, and represents the first study of active fungal communities on grapevine grafted plants by comparing traditional and next-generation sequencing methods.

1. Introduction

Hot-water treatment (HWT) is an efficient, environmentally safe and commercially viable method of suppressing a wide range of pests and pathogens in planting material of grapevine and other crops (Waite and May 2005). For grapevine, it comprises the submersion in water of dormant cuttings, rootlings or grafted rootlings for a given temperature and time (Waite and Morton, 2007; Gramaje et al., 2009). The HWT mechanism is the application of heat to the material in order to denature the pathogens and kill arthropods and nematodes. It is especially effective in controlling crown gall (Ophel et al., 1990; Burr et al., 1996), phylloxera (Buchanan and Whiting, 1991), phytoplasma diseases (Caudwell et al., 1997; EPPO, 2012) and the European quarantine agent *Xylella fastidiosa* (Goheen et al., 1973; Purcell et al., 2013; EFSA PLH Panel, 2015), and reducing the incidence of fungal trunk pathogens (Fourie and Halleen, 2006; Gramaje et al., 2009; Halleen and Fourie, 2016).

Grapevine trunk diseases are caused by a wide range of taxonomically unrelated fungal species that colonize the wood of spurs, cordons, cuttings and trunk, compromising the translocation of

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nutrients and water throughout the vine, which eventually leads to death of the woody tissues. Nurseries are particularly vulnerable to trunk disease infections since propagation of grapevines creates wounds for pathogen invasion as well as the means of spreading inoculum in asymptomatic planting material (Gramaje and Armengol, 2011). Chemical control of these endogenous fungal pathogens inhabiting the vascular tissue of grapevines is difficult. Standard treatments applied to the surface of cuttings to manage other fungal diseases in nurseries do not penetrate the cutting tissue sufficiently to be effective (Gramaje et al., 2018). HWT is, therefore, the only currently recognized means of controlling internal infections of fungal trunk pathogens in propagating material. However, there is a perception in the vine nursery industry that the HWT process sterilizes completely both the surface and the internal wood of cuttings, making the plants more vulnerable to any kind of new fungal infection. Bruez et al. (2017) recently evaluated the long-term impact of HWT on the fungal microbiota and concluded that the initial reduction of fungal communities by HWT did not persist over time. The effects of the changes to the population of internal microorganisms (endophytes and/or fungal pathogens) on cuttings that result from HWT protocols are still unknown.

Fungal communities in plants can be examined by different approaches. Cultivation-based techniques have been frequently used before, however, these methods tend to misrepresent fungal activity and underestimate species richness, because fungi may be hidden, highly selective and slow growing. Molecular-based approaches have progressively replaced morphological approaches to characterize microbial communities in nature. They allow the detection and identification of more microorganisms, including species that cannot be obtained in culture (Amann et al., 1995). The new advances in high-throughput sequencing technology have increased both the resolution and scope of fungal community analyses and have revealed a highly diverse and complex mycobiota of plant vascular systems (Studholme et al., 2011). To date, most studies have investigated the ecology of total fungal communities by sequencing the ribosomal RNA genes (rDNA) (Lindahl et al., 2013), which provide a description of all members of the community, regardless of activity level. For example, DNA based methods are unable to distinguish between viable or dead organisms with intact genetic material (England et al., 1997; Demanèche et al., 2001).

Sequencing the ribosomal DNA transcripts (rRNA) instead reveals the metabolically active fungal taxa of the community and provides insights into their activity in environmental samples (Urich et al., 2008). The study of the potentially active fungal communities by using rRNA sequences has been carried out in environments such as soil (Baldrian et al., 2012; Barnard et al., 2013; Kuramae et al., 2013), decaying plant material (Rajala et al., 2011) and in the atmosphere (Womack et al., 2015), but has not been applied to elucidate changes of fungal communities in the wood of economically important crops, such as grapevine.

In grapevine, the ecology of total fungal communities is so far largely derived from studies on culture-dependent methods (Casieri et al., 2009; Martini et al., 2009; Gónzalez and Tello, 2010; Hofstetter et al., 2012; Pancher et al., 2012; Bruez et al., 2014, 2016, 2017) or cultivation-independent rDNA approaches (Pancher et al., 2012; Bruez et al., 2014, 2016), and information is lacking on the relationships between the diversity of the total community and the community of active microbes. The present study investigated changes in the potentially active fungal communities of internal grapevine wood after HWT in the Czech Republic and Spain. For this purpose, we examined fungal communities in grapevines using both traditional microbiological approach and HTAS of internal transcribed spacer 2 (ITS2) region in extracted total RNA. Anderson and Parkin (2007) indicated that the ITS provides a more active part of the fungal community than 18S rRNA as it is continually transcribed but quickly removed in the processing of mature rRNA.

We tested the following hypotheses: (1) the ITS2 region of rRNA is a suitable marker for revealing active fungal species in a community, (2) only part of the fungal community colonizing the wood of grapevine is metabolically active at a particular HWT temperature/time combination, (3) the HTAS procedure significantly enhances the characterization of fungal diversity compared to traditional methods, (4) metabolic activity of fungal species is related to the origin of grapevine planting material, which changes after one season in the vineyard, (5) HWT process does not sterilize completely the internal wood of cuttings but reduces the infection caused by fungal trunk disease pathogens.

2. Materials and methods

2.1. Planting material and treatments

Two experiments were simultaneously carried out in Spain and the Czech Republic in 2015 to examine the effect of hot-water treatments (HWT) on the total mycobiota of dormant grapevine grafted cuttings. In each country, a stock of 450 dormant grafted plants ready to be sold to producers, cv. Garnacha Tintorera grafted onto rootstock 110 Richter and Sauvignon Blanc grafted on SO4, were obtained from commercial nurseries in Spain and the Czech Republic, respectively. Grafted plants were obtained following the propagation process in nurseries described in Gramaje and Armengol (2011). No chemicals or biocontrol agents were applied during the different stages of the propagation.

In April 2015, this planting material was allocated at random to 3 bundles of 150 grafted plants. One bundle was assigned to no-HWT (control). The remaining two bundles were assigned to either HWT at 50 °C 30 min or HWT at 53 °C for 30 min. For HWT, planting materials were placed in a hydrating bath for 1 h to presoak material before treatment. Following hydration, plants were placed in mesh polyethylene bags and immersed in a temperature controlled bath at the experimental temperatures and times for the treatment. On removal from the HWT bath, vines were immediately plunged into a cool bath of clean water at ambient temperature for 30 min in order to stop the heating process. Vines were then removed from the bath and allowed to drain until there was no free moisture on the surface of the cuttings. Then, two groups of 20 grafted plants were randomly collected from each bundle. For each treatment, one of the groups of 20 grafted plants were subjected to fungal isolation and the other were flash-frozen in liquid nitrogen and subjected to isolation of total RNA followed by HTAS. All HWTs were carried out at the Polytechnic University of Valencia.

The remaining grafted plants (110 per treatment) were planted immediately in a commercial vineyard, following standard cultural practices in each country during the grapevine growing season. At the end of the growing season (October 2015), two groups of 20 grafted plants were randomly collected from each treatment and, again, one of these groups of 20 grafted plants was subjected to fungal isolation and the other to HTAS.

2.2. Fungal isolation and molecular identification

Isolations from Czech and Spanish planting material were performed at the Polytechnic University of Valencia. In each plant, isolations were performed from sections (2 cm long), which were cut from two different areas: the basal end of the rootstock cuttings (crown area) and the grafting area. These sections were then washed under running tap water, surface-sterilized for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Fifteen internal wood fragments per section were