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Investigating the durable effect of the hot water treatment used in nurseries on pathogenic fungi inhabiting grapevine wood and involved in Grapevine Trunk Diseases

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ABSTRACT

Hot water treatment (HWT) is used in nurseries to control pathogenic fungi involved in Grapevine Trunk Diseases (GTDs), as well as other pathogens, such as phytoplasmas. The long-term impact of this treatment on the entire microflora, especially on the general fungal microbiota living inside plants, still remains however unknown. In this study, the fungal microflora of vineyard plants, treated or not by HWT 14 and 15 years earlier were compared at different plant part levels. The fungal microflora was relatively abundant in the different types of wood tissues. Certain fungal genera were first isolated and then identified on the basis of their treatment or not by HWT. A significant change between 2010 and 2011, the two sampling years, was detected. Although the HWT may have affected the cuttings microflora at the nursery stage, this had not persisted after several years of HWT treatment for the fungi, especially the pathogenic ones. As HWT does not have a significant long-term control effect on GTD pathogens in mature plants in the vineyards, applying other sanitary methods as soon as HWT-young vines are planted in the vineyards is recommended.

1. Introduction

Grapevine Trunk Diseases (GTDs), the most predominant vine diseases, are found throughout many of the world's vine-growing countries, such as France, Spain, Italy, Portugal, South Africa, Australia, Chile, USA (California). The causal agents are various fungi, principally *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *Fomitiporia mediterranea, Diaporthe ampelina, Cylindrocarpon* spp. and *Botryosphaeria* spp. (Scheck et al., 1998; Mugnai et al., 1999; Armengol et al., 2001; Rumbos and Rumbou, 2001; Edwards and Pascoe, 2004; Gimenez-Jaime et al., 2006). In France, 13% of vineyards are currently unproductive because of these diseases (Grosman and Doublet, 2012). In certain wine regions, such as Cognac, the percentage of unproductive grapevines was estimated between 2003 and 2008 at 32.6% and, in the Jura, at 18.4% (Bruez et al., 2013). The use of sodium arsenite, the only chemical product capable of reducing such diseases in the vineyards, was banned in 2003 in Europe, due to its toxicity for Human health and the environment. Various treatments have since been tested, with variable success, to control such diseases in the vineyards. Additionally, sanitary measures have to be applied in nurseries to prevent initial plant contamination by pathogenic fungi. Gramaje and Armengol (2011) have suggested that an integrated management programme for grapevine propagation material should be developed. In that programme, Hot Water Treatment (HWT) has been used to prevent pathogen infections in nursery cuttings. Initially, HWT was principally used against Flavescence Dorée and Bois Noir, two Phytoplasma-related diseases, but it also has an impact on GTDs (Chalak et al., 2013). Crocker et al. have equally pointed out that some *Vitis vinifera* L. cultivars, such as Pinot Noir, Chardonnay, Merlot, Riesling and Petit Verdot, and certain rootstock varieties, Ramsey and Ruggeri 140, are naturally sensitive to HWT (Crocker et al., 1999). Laukart et al. (2001) have shown, too, that when HWT is used on Pinot Noir, it influences the dark pith coloration (Waite and May, 2005). HWT can be a practical and

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relatively inexpensive means of controlling fungal pathogens in dormant wood (Caudwell et al., 1997). Nonetheless, negative effects, including the delayed callusing and rooting of HWT cuttings, have been observed by growers (Halleen et al., 2007). Even if HWT does control a number of grapevine pests and diseases in dormant grapevine cuttings and young rooted grapevines (Mannini, 2007), it needs to be carefully applied because it may interfere with the vitality of wood propagation (Bleach et al., 2013). Caudwell et al. (1997) have described that HWT eliminates microorganisms without damaging the grapevines when it is applied at 50 °C for 45 min. In New Zealand and Australia, HWT is usually used during the production of cuttings. HWT is also used to control black-foot disease, caused by *Cylindrocarpon* spp., and Petri disease, associated with *P. chlamydospora* and *P. minimum*. In South Africa, Fourie and Halleen (2004) have shown that infected propagation material is responsible for the dispersal of pathogens that cause young vine decline and that HWT (50 °C for 30 min) could control such infection. In Spain, Gramaje et al. (2009) have shown that, when applied for at least 30 min at 54 °C, HWT had an effect on Petri disease pathogens. In France, Larignon et al. (2008) have shown that, immediately after applying HWT to the cuttings for 50 °C during 45 min, the pathogenic fungi involved in GTDs, such as *P. chlamydospora*, *Diplodia seriata, Diaporthe ampelina,* were not isolated from the wood, but that *P. minimum* and *Neofusicoccum parvum* were isolated (Larignon and Dubos, 1997).

Although HWT can protect nursery cuttings from infection by certain phytopathogens involved in GTDs, there is currently no data regarding its effect on fungal microflora as a whole and over a long period. As growing vines contain a wide range of fungal microflora, HWT might also impact some members of this microbiota.

The aim of this study was to determine whether HWT could have a long-term impact on the pathogenic fungal microflora involved in GTDs 14 and 15 years after the treatment. The specific objectives were to: *(i)* isolate and characterize the fungi colonizing the HWT and non-HWT vines (HWT/Control); *(ii)* compare their respective fungal communities; *(iii)* observe any differences between the microflora colonizing the wood tissues of vines sampled in 2010 and 2011.

2. Materials and methods

2.1. Plant material and sampling

Experimentation was carried out by uprooting grafted Pinot noir grapevines planted in sandy soil in 1996 from a vineyard located in Marsannay-la-Côte in Burgundy (France). The rootstock was 3309 Couderc clone 114, and the scion cultivar a Pinot noir clone 115. Before the grapevines were planted, half of the 2508 plants plotted in 26 ranks had been subjected to HWT (50 °C for 45 min). The plants, which were collected in September 2010 and 2011 were, consequently, either 14 or 15 years old.

2.2. Fungal isolation and identification

Eight HWT and eight control grapevines were sampled. Each plant had expressed leaf stripes symptoms and an apoplectic form, for the first time in 2010 or in 2011. In order to study the fungal microflora colonizing the wood tissues, the plants were cut horizontally and transversally, and the rootstock part, graft union and trunk were separated. Different types of necrosis were then determined as white-rot, black punctuation (xylem vessels becoming black), black dots (zone with a lot of black punctuation), sectorial necrosis and central necrosis. All these tissues were sampled, and distinguished according to whether HWT or Control plants were concerned. Chips of each type of wood tissue were sampled.

2.3. Isolation and identification on agar media

For each type of necrosis, located in the rootstock, graft union or trunk, ten chips ($5 \times 5 \times 1$ mm) of wood were aseptically dissected, before being immerged for 15 s in 3% calcium hypochlorite. The 10 chips were then deposited on two Malt Agar Petri plates amended with chloramphenicol (0.025% w/v) against the bacterial development.

Fungal strain development was monitored for three weeks. Whenever possible, the taxonomic identification of the endophytic fungi was based on morphological and cultural features, combined with an examination of fruiting structures and fungal conidia under the microscope (Leitz LaborLux D, Germany). All isolated fungi were kept in Malt Agar Petri Plates in a collection room chamber at 4 °C.

2.4. Data analysis

2.4.1. Species diversity

Specific diversity indexes, Shannon H, and Simpson D (Shannon and Weaver, 1963; Buckland et al., 2005) were calculated and used to separate the different parts and types of grapevines (HWT and Control vines). Such indexes were estimated using the package Vegan of R version 3.0.2 software.

Comparison of the pathogenic fungal species between the HWT and Control were made using the Shapiro-Wilk test. As the data did not follow the normality, the Wilcoxon test was applied.

2.4.2. Rarefaction curves, diversity estimates

Species accumulation curves were estimated, using the number of species, for all the grapevines studied. The data of the eight HWT and the eight control plants were pooled. Curves were defined using Estimates software.

2.4.3. Canonical correspondence analysis

Canonical correspondence analysis (CCA) was used to determine the effect of the HWT on the fungal communities of the grapevines studied (Bruez et al., 2015). CCA was performed using R software package ade4. Analyses were based on the relative abundance of all the species found in the samples. The sampling year was treated as an independent variable, the species relative abundance as a dependent variable, and HWT or control vines as the co-variable. Centroids for independent variables (sampling dates and HWT or Control), as well as species scores for the fungi, were presented in a biplot plan. Proximity of a species at the centroid signifies that the species has a high relative abundance in the samples.

2.4.4. Non-metric multidimensional scaling (NMDS)

Non-metric multidimensional scaling (NMDS) was used to visualize the similarity level of individual cases in a dataset (Holland, 2008) and to refer to a set of related ordination techniques for information visualization, especially concerning the information contained in a distance matrix. The NMDS were defined using the package Vegan of R version 3.0.1 software. To validate those results, the statistical test ANOSIM was verified using package Vegan of R software.

3. Results

3.1. Status of the wood

When the plants were cut, different types of necrosis were observed, depending on the specific parts (Fig. 1). Necrotic wood