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Screening and modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella chlamydospora* involved in grapevine trunk diseases

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ABSTRACT

The antagonistic activity of 46 bacterial strains isolated from Bordeaux vineyards were evaluated against *Phaeomoniella chlamydospora*, a major grapevine pathogen involved in Esca. The reduction of the necrosis length of stem cuttings ranged between 31.4% and 38.7% for the 8 most efficient strains. Two *in planta* trials allowed the selection of the two best strains, *Bacillus pumilus* (S32) and *Paenibacillus* sp. (S19). Their efficacy was not dependent on application method; co-inoculation, prevention in the wood and soil inoculation were tested. The involvement of antibiosis by the secretion of diffusible and/or volatile compounds in the antagonistic capacity of these two strains was assessed *in vitro*. Volatile compounds secreted by *B. pumilus* (S32) and *Paenibacillus* sp. (S19) were identified by gas chromatography/mass spectroscopy (GC/MS). The volatile compounds 1-octen-3-ol and 2,5-dimethyl pyrazine were obtained commercially and tested, and they showed strong antifungal activity against *P. chlamydospora*, which suggested that these compounds may play an important role in the bacterial antagonistic activity *in planta*.

Furthermore, the expression of 10 major grapevine defense genes was quantified by real-time polymerase chain reaction, which demonstrated that the two strains significantly affected the grapevine transcripts four days after their application on the plants. High expression levels of different genes associated with *P. chlamydospora* infection in *B. pumilus* pre-treated plants suggests that this strain induces systemic resistance in grapevine. For the first time, we demonstrated the ability of two bacterial strains, *B. pumilus* and *Paenibacillus* sp., isolated from grapevine wood, to control *P. chlamydospora via* direct and/or indirect mechanisms.

1. Introduction

Grapevine trunk diseases (GTDs), such as Esca, Eutypiosis and Botryosphaeriae diebacks markedly impact the worldwide wine and grape industry. The heavy economic losses caused by these diseases, especially Esca, indicates that they are becoming a growing threat to grapevine and the production of quality wine wherever grapevines are cultivated (Lorrain et al., 2012). For example in France, approximately 13% of vineyards are unproductive because of GTDs (Bruez et al., 2013).

Symptoms of Esca are wood decay, symptoms on the leaves and brown spots on the berries. Foliar symptoms include leaf chlorosis and necrotic tiger stripes (Lecomte et al., 2012). Symptoms present on the wood are central necrosis, black punctuate necrosis, sectorial necrosis and a discolored xylem stripe and white rot, which is the most common and specific symptom of Esca (Lecomte et al., 2012; Maher et al., 2012). In severe cases, these symptoms result in the death of the plant.

Isolation and identification of the fungi associated with Esca have revealed several pathogenic species, including *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield & L. Mugnai) P.W. Crous & W. Gams (Pch), *Phaeoacremonium aleophilum* (*P. mini-*

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mum com.nov.) (Gramaje et al., 2015) and *Fomitiporia mediterranea* (Fom) M. Fischer (Ciancio and Mukerji, 2008; Surico, 2009). These three species are considered as the major pathogens associated with Esca (Bertsch et al., 2013; Larignon and Dubos, 1997). Nevertheless, other fungal species, *i.e.*, *Eutypa lata, Stereum hirsutum* and *Botryosphaeriaceae*, are also frequently isolated from infected plants and may be associated with Esca to some extent (Larignon and Dubos, 1997; Laveau et al., 2009). Because these pathogens have never been detected in the leaves of infected plants, the foliar symptoms of Esca have been hypothesized to originate from toxin transport from colonized wood to the leaves (Andolfi et al., 2011; Spagnolo et al., 2012) and/or from the disruption of the vessel sap flow (Lecomte et al., 2012). Environmental stress has also been reported as a major influence on the expression of Esca (Luini et al., 2010). Furthermore, wounds due to grapevine pruning are generally considered as the principal port of entry for the fungal pathogens associated with GTDs (Chapuis, 1998; Graniti et al., 2006; Niekerk et al., 2011). *P. chlamydospora* has been consistently isolated from grapevines showing symptoms of Esca. Pathogenicity tests have clearly demonstrated the presence of this pathogen at the origin of typical Esca necrosis lesions in the wood (Laveau et al., 2009). The susceptibility of fresh pruning wounds colonized by *P. chlamydospora* conidia has been reported (Eskalen et al., 2007; Larignon et al., 2000). The pycnidia produced by *P. chlamydospora* on the exposed vascular tissue on the cordons and spurs of grapevines could serve as an inoculum source in the vineyard (Eskalen et al., 2001; Eskalen and Gubler, 2002; Larignon et al., 2000). *P. chlamydospora* has been isolated from rootstock mother plants (Aroca et al., 2009) and from scion cuttings (Zanzotto et al., 2007). The presence of *P. chlamydospora* on nursery vine plants has also been reported by Vigues et al. (2009). In addition, Moyo et al. (2014) suggested that many different arthropods could carry *P. chlamydospora* spores and might serve as an inoculum source.

Sodium arsenate was banned in 2001 due to its human and environmental toxicity and was the only pesticide registered for the control of GTDs; since then, no treatment has been developed that efficiently controls Esca. Therefore, the development of an alternative method such as biocontrol is desirable. Thus, to prevent the spread of GTDs and to reduce the use of pesticides to control grapevine diseases, studies of complementary and/or alternative methods, especially biocontrol, have sparked great interest in viticulture.

As previously reviewed, various microorganisms have been tested to control the fungal pathogens associated with Esca (Bertsch et al., 2013; Compant et al., 2013). Most studies have focused on the biocontrol effect of *Trichoderma* spp. (incl. *T. harzianum, T.atroviride*) against several fungal pathogens related to GTDs (Di Marco et al., 2004; Fourie and Halleen, 2006; Halleen and Lombard, 2010; Kotze et al., 2011; Mounier et al., 2014). The antagonistic potential of various bacterial strains has been explored *in vitro* or on wood disks for the biological control of these fungi in grapevines (Alfonzo et al., 2009; Lebrihi et al., 2009). Furthermore, only a low number of antagonistic bacterial strains have been reported to suppress GTD agents *in planta*, including *P. chlamydospora*, *N. parvum*, *N. australe*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *E. lata*, and *Phomopsis viticola* (Ferreira et al., 1991; Haidar et al., 2016; Kotze et al., 2011). *In vitro* assays have shown that the inhibitory effect of metabolites of *Bacillus subtilis* (AG1) on the growth of *L. theobromae*, *P. chlamydospora* and *P. aleophilum* is efficient (Alfonzo et al., 2009). Moreover, the biocontrol activity of the bacterial strain *B. subtilis* B1a, *Erwinia herbicola* (strains JII/E2 and JII/E4) and the A*ctinomycete* strain A123 was demonstrated *in vitro* and on autoclaved grapevine wood discs against *E. lata* (Schmidt et al., 2001). More specifically, the efficacy of *B. subtilis* for the protection of grapevine against *P. chlamydospora* by reducing pruning wound infections (Kotze et al., 2011) has been shown. In another study, a different strain of *B. subtilis* isolated from grape wood arm has been demonstrated to significantly reduce infection by *E. lata* in grapevine wood (Ferreira et al., 1991).

Lastly, despite several attempts, no commercial bacterial biocontrol products have been developed to control GTDs. For example, only one fungal product (Esquive®) based on *Trichoderma atroviride*, is currently registered in France. Thus, in order to find a suitable biological control agent (BCA) adapted to vineyard conditions, we evaluated 46 bacterial strains, all of which originated from vineyards, either from grapevine wood or grape berries, and all had already been tested against two other major grapevine fungal pathogen species, *Botrytis cinerea* and *N. parvum* (Haidar et al., 2016).

The objectives of this study were (1) to screen the 46 bacterial strains isolated from grapevine for significant antagonism against *P. chlamydospora*, and (2) to identify the mechanisms of action that potentially accounted for the inhibition of the pathogen by the most effective bacterial strains. In addition, we examined the disease control efficacy of selected bacteria by comparing three application methods (*i.e.*, co-inoculation, preventive inoculation in the hole and preventive inoculation by soil drenching).

2. Materials and methods

2.1. Microorganisms and cultural media

2.1.1. P. chlamydospora

P. chlamydospora strain (SO44) was selected from the INRA-UMR 1065 SAVE collection, Bordeaux and was used in all experiments. This strain was originally obtained in 1996 from a Cabernet Franc cultivar in Moncaup, France. It was characterized as highly aggressive in previous studies at INRA (Laveau et al., 2009). The strain was subcultured on Malt Agar (MA) medium and incubated at 22 °C (12 h light/12 h dark) for one month before being utilized for artificial inoculation in bioassays with cuttings and for two weeks for *in vitro* tests (confrontation and volatiles).

2.1.2. Bacterial strains

A total of 46 bacterial strains were tested which were derived from a previous study, *i.e.*, Haidar et al., 2016 (see Table S1 in Supplementary material in the online version at DOI: 10.1016/j.micres.2016.07.003). All the strains were isolated from grapevine, including 35 strains from wood tissue (Bruez et al., 2015) and 11 strains from the grape berry surface (Martins, 2012). In these two last articles, the bacterial strains were typed. The strains from the grape berry surface originated from the "Centre de Ressources Biologiques en Oenologie" (University of Bordeaux and Bordeaux INP "Institut National Polytechnique of technology"). For the *in vitro* trials, strains were grown beforehand on Trypto-Casein Soy Agar medium (TSA, Biokar diagnostics, France) for 24 h at 28 °C. In both 2013 and 2014 bioassays, the bacterial preparation was done as described in Haidar et al., 2016.

In the 2015 bioassay, the bacterial cell concentration of S19 and S32 was adjusted to 10^8 CFU/ml for co-inoculation. For preventive inoculation by soil drenching and in the hole, the bacterial cell concentration of *Paenibacillus* sp. (S19) and *B. pumilus* (S32) was adjusted to 10^7 and 10^6 CFU/ml, respectively.

2.2. Stem disease bioassays

2.2.1. Bacterial and fungal inoculation treatments

In all bioassays, each cutting stem was surface-sterilized by rubbing it with a paper towel soaked with 95% ethanol. Then an artificial wound was made by drilling a hole in the bark (4 mm in