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Pre-selection in laboratory tests of survival and competition before field screening of antagonistic bacterial strains against *Botrytis* bunch rot of grapes

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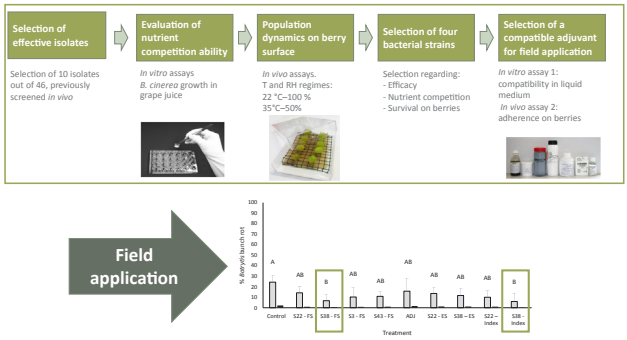
GRAPHICAL ABSTRACT



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ABSTRACT

With only a few biocontrol products currently registered against *Botrytis* bunch rot (BBR) of grapes, there is a crucial need for new antagonistic strains that are able to survive and efficiently suppress *B. cinerea* under vineyard conditions. The aim of this study was to establish and follow a pre-selection process among potential antagonistic bacterial strains, previously identified *in vivo* for efficacy, and to carry out a further field screening assay using a reduced strain number. Ten bacterial strains were pre-selected and tested, *in vitro* and *in vivo*, to characterise their mode of action and population dynamics under simulated climatic regimes. Four candidate strains were then selected and characterised for high efficacy *in vivo*, known mode of action and marked survival ability. Some suitable additives for increasing strain adherence on grape berry surface were tested prior to field applications, indicating one commercial adjuvant for potential improved bacterial persistence in the field. The four strains were applied separately in an experimental Merlot vineyard near Bordeaux (SW France), either at five key phenological stages, or following a specially developed Disease Risk Index (DRI). The *Bacillus ginsengihumi* S38 strain treatments significantly reduced BBR incidence by 72–75% compared to the control, whereas sprays applied according to the DRI decision support system tended to improve disease control. The study validates a laboratory pre-selection process followed by a field screening step, resulting in a candidate *B. ginsengihumi* strain S38 with a high potential for BBR biocontrol and future development in vineyards.

Abbreviations: BBR, *Botrytis* bunch rot; BCA, biological control agent; CFU, colony forming units; DRI, disease risk index; MoA, mode of action; RH, relative Humidity; SDW, sterile distilled water; T, temperature

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1. Introduction

Biological control of fruit pathogens with microbial antagonists is regarded as a disease management strategy alternative to the use of synthetic fungicides, with a high potential to control fruit crop diseases (Nicot et al., 2011; Romanazzi et al., 2016). In this context, many studies have investigated, using *in vitro* or *in vivo* experiments, biological control of *Botrytis cinerea* Pers.:Fr., the causative agent of grey mould affecting economically important crops such as tomatoes, apples, strawberries or grapes (Ballet et al., 2016; Marín et al., 2016; Passera et al., 2017; Qin et al., 2017; Ruiz-Moyano et al., 2016; Sylla et al., 2015; You et al., 2016). In vineyards, the disease, also known as *Botrytis* bunch rot, represents, mostly in temperate climate regions, a major challenge for yield and wine quality (Ky et al., 2012). In the last ten years, several studies have shown new advances in biological control strategies against BBR, by developing either new biological control agents, or new application strategies using yeasts, filamentous fungi and/or bacteria prior to harvest (Calvo Garrido et al., 2017; Haidar et al., 2016c; Parry et al., 2011). In addition, only a few commercial products, based on fungal or bacterial genera, are available in Europe for biological control of BBR (Nicot et al., 2016). However, the control rates performed by these products tend to vary between orchards and from one season to the next. Consumers also increasingly demand zero-residue fruit goods and wines. However, despite more than 30 years research in biological control, reliable commercial solutions to control BBR are still lacking, indicating that greater research effort are still needed to develop new BCAs, adapted to the vineyard environment and effective in those field conditions, with lower variability in disease control. Recent research in INRA Bordeaux-Aquitaine has evaluated the efficacy of up to 46 bacterial strains against *B. cinerea* infection, using *in vitro* and *in vivo* biotests (Haidar et al., 2016a). As these strains were originally isolated from grape berry surface or grapevine wood (Bruez et al., 2015b; Martins et al., 2013), this may be considered an *a priori* advantage, possibly allowing these BCA bacterial strains to better survive and compete in this particular crop ecosystem, following their introduction in vineyards.

The mode of action of a BCA strain is one of the most important features to be investigated and understood in order to i) better analyse efficacy results and those factors which may interfere with efficacy, and ii) determine optimal BCA application strategy in the field, and the possibility to include it in combinational strategies (Haidar et al., 2016c; Spadaro and Droby, 2016). Many studies in biocontrol literature have shown disease reductions under *in vitro* and/or *in vivo* conditions, thereby suggesting new microbial candidates. Due to economic, technical and time constraints, only a few studies have also tested the BCA candidates in the field, with biocontrol efficacy often being markedly reduced, even lost, compared to laboratory experiments (Köhl et al., 2011; Nicot et al., 2011). The major reason for such an unsuccessful outcome comes mainly from the difficulties of BCA populations to adapt to, and survive on, the aerial host organs (phyllosphere or carposphere) under field environment conditions (Köhl et al., 2011; Nicot et al., 2011). Consequently, specific studies evaluating the survival ability of BCA candidates, MoA and their suppressive efficiency under different conditions should be included in preliminary laboratory experimental steps before subsequent field screening. Other strategies may also improve survival ability and population persistence after field application, e.g. the use of adjuvants that may improve cell adherence to fruit surfaces and favour BCA establishment on leaves, flowers and/or berries (Ballet et al., 2016; Calvo-Garrido et al., 2014b; Di Francesco and Mari, 2014; Marín et al., 2016).

A systematic stepwise screening process has been proposed by Köhl et al., (2011) as a general framework procedure for any biological control pathosystem. Following these guidelines may be very useful to achieve a short, efficient and field-oriented selection process of strains that are also adapted to specific pathosystem or biological control conditions. In this context, the present study is aimed at developing such a selection process for the bacterial strain collection at INRA Bordeaux-Aquitaine (Haidar et al., 2016a; Haidar et al., 2016b). Although a single BCA candidate with appropriate characteristics may prove sufficient in a general screening process, including more than one BCA candidate in field trials could also maximise success opportunities. Doing so might include candidate strains with lower laboratory efficacy, but with other potential advantages. Selecting and developing a microbial BCA is a long and costly process in which the likelihood of successful strains becoming commercial products is extremely low (Köhl et al., 2011). Well adapted pre-selection procedures are, therefore, needed to maximise success probabilities and shorten this screening process, providing practical solutions for growers.

The major objectives of this work were, firstly, to characterise some key life traits of ten pre-selected bacterial strains, effective against *B. cinerea*, to assess their potential as BCAs in vineyards. The life traits included nutrient competition ability, antibiosis aptitude based on diffusible compounds, survival under simulated climatic regimes, and compatibility with spray adjuvants. The second objective was to select four of those strains with high biocontrol potential, to be tested and compared in a one-season field screening trial, by assessing their efficacy against BBR and their survival capacity under Bordeaux region climatic conditions.

2. Materials and methods

2.1. B. cinerea strain used in laboratory experiments

The *B. cinerea* pathogenic strain (code = 213), selected from the INRA-UMR 1065 SAVE collection in Bordeaux, belongs to the *transposa* genotype and has been characterised as highly virulent on grapevine berries at different stages (Deytieux-Belleau et al., 2009; Martinez et al., 2005). Routine cultures were maintained on malt agar medium ($15\,g\,L^{-1}$ of malt from Biokar Diagnostics and $20\,g\,L^{-1}$ of Setaxam® agar) at 22 °C. In order to obtain a conidial suspension, *B. cinerea* conidia were collected by adding SDW to sporulating cultures on malt agar medium plates and gently rubbing with a sterile spatula. Concentration of the conidial suspension was determined using a haematocytometer, and then diluting to adjust to the desired final concentration.

2.2. Bacterial strains and pre-selection of ten effective BCA candidate strains

Bacterial isolates were all originally isolated from grapevine tissues (Bruez et al., 2015a; Martins et al., 2013) and were maintained in the collections of either INRA Bordeaux-Aquitaine or “Biological Resources Center for Enology” (University of Bordeaux and Bordeaux Polytechnic Institute). These strains were characterised in previous studies (Haidar et al., 2016a; Haidar et al., 2016c; Haidar et al., 2016d). The strains were maintained on cryogenic storage beads (Viabank MWE, Wiltshire, England) at −20 °C, then subcultured on TSA solid medium (Trypto-casein Soy Agar; Biokar Diagnostics, Beauvais, France) and incubated at 27 °C in the dark, before use. Liquid cultures were obtained by inoculating bacterial colonies from TSA medium in Erlenmeyer flasks containing TSB liquid medium (Trypto-casein Soy Broth; Biokar Diagnostics, Beauvais, France), and then incubated at 27 °C for 48 h using an orbital shaker at 150 rpm. Liquid cultures were then centrifuged in sterile centrifuge flasks at 5000 rpm during 10 min and then re-suspended in phosphate buffer to obtain the liquid suspensions used in laboratory and/or field experiments. The bacterial strain populations in suspension were quantified by fluorochrome staining (500 µL Chemsol B16 buffer + 2,5 µL de fluorochrome Chemchrome V6 fluorescein acetate; Biomérieux, Marcy l’Etoile, France) followed by epifluorescent direct counts using an optical microscope (Model BH2, Olympus France, Rungis, France). A minimum of 300 cells were counted in at least 10 different fields of view, and the average number of dyed cells