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Bacillus Velezensis as Antagonist Towards *Penicillium Roqueforti s.l.* in Silage: *In Vitro* and *In Vivo* Evaluation

Eva Wambacq[1,2,*], Kris Audenaert[1], Monica Höfte[1], Sarah De Saeger[3] and Geert Haesaert[1,2]

[1] Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Belgium

[2] Department of Biosciences and Food Sciences, Faculty of Science and Technology, University College Ghent, Belgium

[3] Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Belgium

Running headline : *B. velezensis* against *P. roqueforti*

* Correspondence: Eva Wambacq, Research station Bottelare (co-management Ghent University and University College Ghent), Belgium. E-mail: eva.wambacq@ugent.be.

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ABSTRACT

Aims: The present study was conducted to evaluate the antagonistic effect of *Bacillus velezensis* NRRL B-23189 towards *Penicillium roqueforti s.s.* and *P. paneum* (designated together as *P. roqueforti s.l.*) in silage conditions.

Methods and Results: Corn silage conditions were simulated *in vitro*, and the impact of *B. velezensis* culture supernatant or cell suspension on *P. roqueforti s.l.* growth and roquefortine C production was evaluated. The antagonism was promising, but growth of *B. velezensis* in corn silage infusion was poor. Additionally, an *in vivo* experiment with microsilos containing a mixture of perennial ryegrass and white clover artificially inoculated with *P. roqueforti s.l.* was carried out. The applied *B. velezensis* cell suspension was unsuccessful in reducing *P. roqueforti s.l.* numbers, but did not compromise the silage acidification.

Conclusions: Although the antagonism observed *in vitro* created high hopes, the applied *B. velezensis* cell suspension could not live up to the expectations *in vivo*.

Significance and Impact of the Study: To our knowledge, the present study is the first one evaluating the antagonistic properties of *B. velezensis* towards toxigenic fungi in silage conditions, offering a good kick-off for further research.

Keywords: antagonism; *Bacillus velezensis*; *Penicillium roqueforti*; silage; roquefortine C

INTRODUCTION

In Belgium, silages are often infected by the toxigenic fungal species *Penicillium roqueforti sensu stricto* (*s.s.*) and *P. paneum*. These two fungal species, referred to as *P. roqueforti sensu lato* (*s.l.*), are very closely related and well adapted to silage conditions. Mainly during the feed-out period of silages, when air inevitably regains entrance into the silo, the prevention of their growth and possible mycotoxin production is difficult (Auerbach *et al.* 1998, Boysen *et al.* 2000, Garon *et al.* 2006, Mansfield and Kuldau 2007, Nout *et al.* 1993, O'Brien *et al.* 2007, Richard *et al.* 2007, Wambacq 2017).

In the field, growth and mycotoxin production by toxigenic fungi can be prevented by good field management and crop husbandry (Boudergue *et al.* 2009, Cleveland *et al.* 2003, Codex Alimentarius Commission 2002, Jouany 2007, Kabak *et al.* 2006), and additionally by the introduction of non-toxigenic isolates of particular fungal species (Cotty and Bhatnagar 1994, Dorner and Lamb 2006, Yiannikouris and Jouany 2002). To obtain high quality silage, application of good silo management is crucial (Dolci *et al.* 2011, Dunière *et al.* 2013, McDonald *et al.* 1991, O'Brien *et al.* 2007, Wilkinson and Davies 2012). As a tool to guide the fermentation process into the desired direction, several types of silage additives are commercially available: fermentation inhibitors (e.g. propionic acid), fermentation stimulants (e.g. homofermentative lactic acid bacteria), aerobic deterioration inhibitors (e.g. heterofermentative lactic acid bacteria), etc. Silage additives inhibiting aerobic deterioration may inhibit growth of both yeast and fungi during the feed-out period. Additionally, antagonistic LAB, yeasts and *Bacilli* have been described (Gourama and Bullerman 1995, McDonald *et al.* 1991, Ongena and Jacques 2008, Oude Elferink *et al.* 2000, Petersson and Schnürer 1995, Schnürer and Magnusson 2005, Wilkinson 2005).

Besides the production of organic acids, LAB can inhibit mycotoxin production by fungi through microbial competition, depletion of nutrients, low pH and production of heat-stable low molecular weight metabolites. The genus *Lactobacillus* is well known for its antifungal activity. Lavermicocca *et al.* (2000) found that a ten-fold concentrated culture filtrate of *L. plantarum* 21B possesses efficient antifungal activity against *P. roqueforti s.s.*, *Aspergillus niger*, *A. flavus* and *Fusarium graminearum*, while Ström *et al.* (2002) described *in vitro* broad-spectrum antifungal activity of *L. plantarum* MiLAB 393 against *F. sporotrichioides* and *A. fumigatus*, but not against *P. roqueforti s.s.*.

Yeasts are well-described antagonists of spoilage fungi *Pichia anomala, P. guilliermondii* and *Saccharomyces cerevisiae* yeasts have been identified for the biocontrol of fungal growth in ensiled high-moisture cereals. Besides competition for nutrients, the antifungal activity of yeasts can be ascribed to the production of cell wall degrading (Droby *et al.* 1989, Jijakli and Lepoivre 1998, Petersson and Schnürer 1995).

The production by *Bacillus* species of compounds that display antifungal activity is well documented (Munimbazi and Bullerman 1998, Pusey 1989, Zuber *et al.* 1993). In plant production, *Bacillus* species including *B. velezensis* have been proven to be promising biocontrol organisms towards fungi infecting growing plants. They can produce cyclic lipopeptides, exerting antifungal effects both in alkaline and acidic conditions. The main families of lipopeptides comprise iturins, surfactins and fengycins, all three exhibiting surfactant properties and antifungal activity (Nam *et al.* 2009, Ongena and Jacques 2008, Romero *et al.* 2007, Velmurugan *et al.* 2009).

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Chitarra *et al.* (2003) demonstrated that an antifungal compound in the culture supernatant of *B. velezensis* NRRL B-23189, able to produce all three families of lipopeptides, inhibits the germination of *P. roqueforti s.s.* conidiospores *in vitro*. Inhibition of conidiospore germination is a beneficial characteristic of an antagonistic organism, since germination is the starting event of a fungus' asexual life cycle (Samson *et al.* 2002).The present study describes both an *in vitro* and *in vivo* experiment aiming to evaluate the antagonistic effect of *B. velezensis* strain NRRL B-23189 towards *P. roqueforti s.s.* and *P. paneum*. *In vitro*, corn silage conditions were simulated in corn silage infusion, while for the *in vivo* experiment a mixture of perennial ryegrass and white clover was artificially contaminated with *P. roqueforti s.s.* or *P. paneum* and treated with *B. velezensis* cell suspension prior to ensiling in microsilos. The *in vitro* experiment revealed promising antagonism of *B. velezensis* towards *P. roqueforti s.l.*, but *in vivo* the bacterial cell suspension could not successfully reduce *P. roqueforti s.l.* numbers in silage. However, it must be noted that the microsilo experiment simulated the silage fermentation process, but not the feed-out of the silage. As an aerobic micro-organism, *B. velezensis* may be able to grow and produce lipopeptides during the feed-out period, but this hypothesis remains to be assessed.

MATERIALS AND METHODS

P. roqueforti s.l. conidiospore suspensions

Two fungal isolates were used for the experiments: *P. roqueforti s.s.* MUCL 46746 (PR) and *P. paneum* CBS 112295 (PP). For the conidiospore suspension preparation, monoconidial fungal inoculum is seeded in the center of 90-mm diameter Petri dishes with Potato Dextrose Agar (PDA, Sigma) in a laminar flow cabinet. The plates are aerobically incubated upright in the dark at 25 °C during 14 days. Conidia were harvested by washing with physiological water (0.85% sodium chloride, Sigma) containing 0.01% Tween 80 (Duchefa), with the aid of a sterile pipet tip. The suspension was transferred to a sterile centrifugation tube. After centrifugation at 8 500 rpm during 15 min, the supernatant was discarded and the conidiospore pellet was resuspended in sterile physiological water without Tween 80 added. The centrifugation and resuspension steps were repeated, after which the conidiospore suspension was filtered through a double layer of sterile miracloth (Millipore) to remove mycelial fragments and conidial aggregates. Conidiospore concentration was determined with a Bürker chamber and adjusted to the desirable concentration with sterile physiological water. When conidiospore suspensions were not readily used, 50% glycerol (Scharlau) was added and the suspensions were stored long-term at -80 °C.

In vitro experiment

Corn silage infusion

Whole crop maize silage (350 gram DM kg^{-1} FM, no roquefortine C detectable, ensiled for 50 days) was dried at 60 °C and milled to 1-mm particles. Corn silage infusion (CSI) was prepared as described by (Niderkorn, 2007): milled maize silage was infused in distilled water at 6 % (w/v) during two hours at 60 °C, followed by filtration through miracloth (Millipore) and a folded paper filter (Whatman 597 ½). After centrifugation at 10 000 rpm during 15 min, the pellet was discarded and the supernatant was collected. The pH of the supernatant was brought to the original pH of the silage (i.e. 3.79) using the same ratio of lactic acid (min. 99%, Fluka) to acetic acid (min. 99%, Sigma) as found in the silage (i.e. 3.17). The supernatant was subsequently sterilized through a syringe filter (cellulose acetate - 0.45 µm pore size - 25 mm diameter, GVS) and stored at 4 °C until use within 24 hours.

B. velezensis treatment solutions

B. velezensis strain NRRL B-23189 (Bv) was grown aerobically in the dark at 30 °C on Plate Count Agar (PCA, Sigma). Four-day old cultures were subcultured in duplo in 15 ml of Brain-Heart Infusion broth (BHI, Sigma) and aerobically stir-cultured in the dark at 30°C on a magnetic shaker at 130 rpm. Additionally, an additional 15-ml portion of sterile BHI was not inoculated with Bv. After 48 hours, one BHI-replicate inoculated with Bv was centrifuged during 15 min at 9 500 rpm. The Bv supernatant was collected and sterilized through a syringe filter (cellulose acetate - 0.45 µm pore size - 25 mm diameter, GVS). The other inoculated BHI-replicate was used as such, as Bv cell suspension (containing $8*10^7$ cfu ml^{-1}), as determined by streak-plating of a dilution series in physiological water on PCA. Sterile BHI was used as a negative control.

Experimental protocol

By combining different volumes of sterile BHI, Bv supernatant or Bv cell suspension with CSI, nine different liquid culture media were prepared (vol/vol): 1) 100 % CSI, 2) 90 % CSI and 10 % sterile BHI, 3) 90 % CSI and 10 % Bv supernatant, 4) 90 % CSI and 10 % Bv cell suspension, 5) 75 % CSI

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and 25 % sterile BHI, 6) 75 % CSI and 25 % Bv supernatant, 7) 75 % CSI and 25 % Bv cell suspension, 8) 50 % CSI and 50 % sterile BHI, and 9) 50 % CSI and 50 % Bv supernatant.

The nine culture media were distributed *in duplo* into two sets of eighteen 15-ml falcon tubes, for infection with either PR or PP to a final conidiospore concentration of $1*10^4$ conidiospores ml^{-1} medium. The first set of falcon tubes was filled with three ml of medium and was used for a microtiter plate assay, as well as for monitoring of conidiospore germination and conidiospore survival. The second set of falcons was used for screening of ROC production. The empty weight of these falcons was noted to allow calculation of the freeze-dried mycelium weight, and all falcons were filled with one ml of medium.

For the microtiter plate assay, 200 µl of culture medium was introduced per well (N=4 per object, except for 90 % CSI + 10 % sterile BHI and 75 % CSI + 25 % sterile BHI: N = 8). Two negative controls without *P. roqueforti s.l.* conidiospores (i.e. 90 % CSI + 10 % Bv cell suspension and 75 % CSI + 25 % Bv cell suspension) were included to enable monitoring of solely Bv growth in CSI, providing insight into its possible use as a silage inoculant. After sealing of the microtiter plate with respiratory foil, the plate was statically incubated in aerobic conditions in the dark for five days at 20 °C. The optical density was determined spectrophotometrically at 620 nm immediately after five days, with subtraction of the initial OD_{620} value per well immediately after filling.

Conidiospore survival in the different culture media was monitored after 24 hours by streak-plating 100-µl samples from the first set of falcon tubes (N=3) on PDA supplemented with 0.5% acetic acid (min. 99%, Sigma). All plates were aerobically incubated bottom-up in the dark at 20 °C and fungal development was evaluated after four days.

Conidiospore germination was evaluated after 24 hours of incubation on 20-µl samples (N=4) taken from the first set of falcon tubes. Per replicate, a 20-µl sample was placed on a glass slide cleaned with 70 % ethanol (Sigma), followed by covering with a clean cover slide and flame fixation. Randomly, 100 conidiospores were counted (evenly spread over the glass slide) and the percentage of germinated conidiospores was determined, using a phase-contrast microscope at 400x magnification (Motic). Conidiospores were considered to have germinated when the length of the germ tube exceeded one-half of the spore diameter.

The second set of falcon tubes was statically incubated in aerobic conditions during five days in the dark at 20 °C without shaking, and stored at -20 °C prior to freeze-drying with an Alpha 1-2 LD Plus lyophilizer (Christ) according to the manufacturer's guidance. After registration of the freeze-dried weight, ROC was quantified by LC-MS/MS (N=1).

Quantitative screening of roquefortine C production

Roquefortine C (ROC) was extracted with ethyl acetate and dichloromethane (both min. 99.5%, Acros Organics) as described by Delmulle (2009), and quantified based on the method described by Monbaliu *et al.* (2010). LC-MS/MS analysis was performed with a Waters Acquity UPLC system coupled to a Micromass Quattro Premier XE triple-quadrupole mass spectrometer (Waters), equipped with Masslynx software for data processing. A 150 mm x 2.1 mm reverse-phase C18 column was used, with a 10 mm x 2.1 mm guard column of the same material (resp. 5 and 3.5 µm inner diameter, Waters). The column was kept at room temperature. The mobile phase consisted of variable mixtures of mobile phase A (water/methanol/acetic acid, 94/5/1 (v/v/v) and 5 mM ammonium acetate) and mobile phase B (methanol/water/acetic acid, 97/2/1 (v/v/v) and 5 mM ammonium acetate) at a flow rate of 0.3 ml min^{-1} with a gradient elution program, mentioned in Table 1.

The injection solvent consisted of mobile phase A/mobile phase B (60/40, v/v) and 5 mM ammonium acetate. The injection volume of the samples on the analytical column was 20 µl. The mass spectrometer was operated in the positive electrospray ionization (ESI+ mode). Capillary voltage was 3.2 kV. High-purity nitrogen was used as drying and ionization (ESI+) nebulizing gas, and argon was used as collision gas for collision-induced dissociation. Source and desolvation temperatures were set at 150 and 350 °C respectively. ROC was analyzed using selected reaction monitoring (SRM). The method was validated according to Commission Decision 2002/657/EC. ROC is expressed relatively to a known amount of internal standard, added to all samples: 0.2 ng of zearalanone (ZAN). To allow quantification of ROC, four reference samples (i.e. ethyl acetate) were spiked with ROC (negative control and 3 known amounts). Based on the response factors (i.e. peak area for ROC / peak area for ZAN) for these reference samples, a linear regression was determined per run and used for the quantification of ROC in the experimental samples. ROC eluted after approx. 7.8 min, while ZAN had a retention time of approx. 9.2 min. In Masslynx, the peak areas for both mycotoxins was determined and the response factor was calculated for each sample. Based on the four spiked reference samples per run, a linear regression was fitted for quantification of the samples with unknown ROC content. The decision limit was 5 ng ml^{-1}, while quantification was possible from 10 ng

ml^{-1} injection solution. Quantification of ROC by LC-MS/MS comprised quantification of predominantly ROC, in combination with its stereo-isomer formed under acidic, basic or photochemical conditions, and with roquefortine D (Richard *et al.* 2004).

***In vivo* experiment**

For the *in vivo* experiment, microsilos with a content of 2.75 liter were use, equipped with a CO_2 slot preventing air ingress but allowing fermentation gasses to escape. Every microsilo has a unique number for identification (Wambacq 2017).

A mixture of perennial ryegrass and white clover (second cut) was mown and prewilted in the field to 420 gram dry matter (DM) per kg fresh matter (FM). After chopping with a New Holland precision chopper to 10-12 cm particles, the starting material was homogenized well prior to ensiling of the different objects: 1) no infection, no additive, 2) PR, no additive, 3) PP, no additive, 4) PR, Bv cell suspension, and 5) PP, Bv cell suspension.

P. roqueforti s.l. conidiospore suspensions were freshly prepared as described previously.

Bv cell suspension was obtained after a three-days incubation period of *B. velezensis* NRRL B-23189 in 100 ml of BHI at 30 °C on a rotary shaker at 130 rpm. After centrifugation at 10 000 rpm during 5 min, the supernatant was discarded and the bacterial pellet was resuspended in 50 ml physiological water containing 16 % glycerol (Scharlau) and stored at -80 °C. Just before ensiling, the cell suspension was defrosted at 20 °C. The concentration of the obtained Bv cell suspension was determined by streak-plating 100-µl aliquots of a decimal dilution series on PCA (N=3). PCA plates were incubated aerobically at 30 °C for four days and *B. velezensis* was enumerated, taking the appropriate dilution factor into account: $5*10^5$ cfu of *B. velezensis* were present per milliliter.

Per object, the fresh feed commodity was spread evenly in a thin layer onto a polyethylene sheet and sprayed with an equal amount of treatment solution using handheld sprayers. Object 1 was sprayed with 20 ml of sterile physiological water per kg FM. Per kg FM, 10 ml of PR or PP conidiospore suspension was applied to objects 2 to 5 (infection at 500 conidiospores per gram FM), as well as 10 ml of the appropriate additive solution: either sterile physiological water (objects 2 and 3), either Bv cell suspension (objects 4 and 5, infection at 5 000 cfu per gram FM). The microsilos were filled in two stages using a pneumatic press. The empty weight of each microsilo was noted before filling and after filling to determine the mean silo density. After 56 days, the microsilos were desiled. The upper and lower 3-5 cm of silage was removed, and the remaining silage was homogenized prior to sampling for determination of pH (Ohmomo *et al.* 1993) and dry matter content (by air draying at 65 °C) and for *P. roqueforti s.l.* enumeration.

To enumerate the amount of *P. roqueforti s.l.* propagules in a sample, exactly 20 grams of fresh matter was brought in a stomacher bag with lateral filter (Interscience) along with 90 ml of sterile physiological water supplemented with 0.01 % Tween 80. After placing the bag in a stomacher (Seward) for homogenization during 1 min at 200 rpm, a decimal dilution series was prepared in physiological water. From this dilution series, 100 µl was streak-plated on Petri dishes containing PDA supplemented with 0.5% acetic acid (O'Brien *et al.* 2008), using a sterile Drigalsky spatula (N=3 per dilution). The plates were aerobically incubated bottom-up in the dark at 20 °C. After five days of incubation, the number of *P. roqueforti s.l.* propagules was counted at the appropriate dilution (i.e. propagule number below 50 per plate). The mean value of the three readings was determined and the number of fungal propagules in the original sample was calculated taking the particular dilution factor into account. *P. roqueforti s.l.* counts were transformed to a logarithmic scale.

Statistical analysis

The obtained data were statistically analyzed with the SPSS Statistics 24 program. Significance was declared at 95%, with $p < 0.05$. Per parameter, normality was checked by Shapiro-Wilk's test (applying Bonferroni correction) and homoscedasticity was checked with Levene's test. A multiple Anova was performed to check for significant interaction between factors. In case of significant interaction, an Anova analysis was performed for each level of one factor to assess the effect of the other factor's). If variances were equal for normally distributed variables, Anova with Tukey as *post hoc* test was performed, otherwise a Welch Anova with Dunnett T3 as *post hoc* test was executed. Not normally distributed parameters were subjected to a non-parametric test according to Kruskal-Wallis with Dunn's test for pairwise comparisons (applying Bonferroni correction). In case of no significant interactions between factors and homoscedasticity, the main effects of the factors were determined likewise over all levels of the other factor(s).

RESULTS

In vitro experiment

Microtiter plate assay for growth monitoring

The results of the microtiter plate assay to monitor *P. roqueforti s.l.* growth are presented in Table 2.

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Over the nine culture media, PR and PP exhibited a different growth pattern. PR showed the highest OD_{620} on 75 % CSI with 25 % sterile BHI. The difference was not significant with 100 % CSI and with the 90 % and 50 % CSI complemented with sterile BHI, but was significant with all the media containing Bv supernatant or cell suspension. The OD_{620} of PP was the highest on 50 % CSI with 50 % sterile BHI, but just like for PR the differences were only significant with media containing Bv supernatant or cell suspension.

Per culture medium, only few differences in growth were detected between PR and PP. On 100 % CSI, PP grew significantly stronger than PR during the five days incubation period, while on 75 % CSI with 25 % sterile BHI the opposite was observed.

In the microtiter plate assay, Bv growth in CSI supplemented with 10% or 25% Bv cell suspension has also been monitored: the mean OD_{620} values were respectively 0.002 and 0.014 for 10 and 25% Bv cell suspension, so growth of Bv cells was poor.

Conidiospore survival and spore germination

Conidiospore survival in the different culture media was evaluated by streak-plating after 24 hours of incubation. Photographs were made of the obtained plates, shown in Figure 1.

Conidiospore survival varied considerably among the culture media: 90 % CSI in combination with 10 % of Bv supernatant facilitated the highest conidiospore survival, followed by 90 % CSI and 10 % Bv cell suspension. Inclusion of 25 % or 50 % of Bv supernatant or cell suspension in the medium resulted in lower conidiospore survival. A striking observation is that the PP conidiospores exhibited stronger growth on the streak-plates compared to PR conidiospores, confirming a lower growth rate of *P. roqueforti s.s.* (Frisvad and Samson 2004, Wambacq 2017).

The results of the conidiospore germination evaluation are presented in Table 3.

For both PR and PP, the highest percentage of conidiospore germination after 24 hours was observed in 100 % CSI, illustrating their good adaptation to silage conditions. In CSI with Bv cell suspension added, in general low germination percentages were observed. Surprisingly, an increased conidiospore germination percentage for PR as well as PP was observed in 90 % CSI with 10 % Bv supernatant, but the difference with 90 % CSI with 10 % sterile BHI was not significant.

PR and PP conidiospore germination only differed significantly after 24 hours of incubation in 75 % CSI complemented with 25 % sterile BHI or 25 % Bv cell suspension: in both media, conidiospore germination of PP was significantly lower compared to PR.

Screening of roquefortine C production

The antagonistic effect of Bv against *P. roqueforti s.l.* has been confirmed on multiple levels (i.e. conidiospore germination and survival, and fungal growth), rendering the tested Bv strain an interesting candidate silage inoculant for *in vivo* inhibition of *P. roqueforti s.l.* growth in silages. However, it must be checked that growth inhibition of *P. roqueforti s.l.* by Bv does not trigger an increased mycotoxin production. Therefore, a quantitative screening of the production of the indicator mycotoxin roquefortine C (ROC) (Auerbach *et al.* 1998) during the five-days incubation period has been performed. These results are presented in Table 4.

Addition of Bv supernatant or cell suspension generally did not result in elevated ROC production by any of the two *P. roqueforti s.l.* isolates compared to CSI in combination with sterile BHI. PP clearly produced less ROC than PR during the five-days incubation period. For PP, the highest ROC levels were detected in 75 % CSI with 25 % sterile BHI or Bv cell suspension, but ROC levels were not highly variable across the nine culture media.

For PR, addition of 10 % sterile BHI, Bv supernatant or cell suspension to 90 % CSI increased ROC production compared to 100 % CSI. Addition of 25 % sterile BHI intensified ROC production even further, while addition of 50 % BHI resulted in lower ROC levels compared to 25 % BHI. The highest ROC production by PR was detected on 75 % CSI with 25 % sterile BHI, which was also the culture medium exhibiting the strongest growth. Sterile BHI is a very nutritious culture medium. Due to Bv growth, the nutrient levels in the BHI in which the bacterium was cultured prior to the start of the experiment have dropped. Therefore, CSI supplemented with Bv supernatant or cell suspension contained less nutrients available for *P. roqueforti s.l.* growth and mycotoxin production. Since a positive correlation between growth of *P. roqueforti s.s.* and ROC production has been detected by Boichenko *et al.* (2002), this might explain the elevated ROC production by PR observed in the media containing 25 and 50 % sterile BHI: on these media the highest growth was registered in the present study.

***In vivo* experiment**

The microtiter plate assay of the *in vitro* experiment showed poor growth of Bv cell suspension in CSI. To check if Bv cell suspension as a silage inoculant would be able to grow and produce cyclic lipopeptides reducing *P. roqueforti s.l.* numbers *in vivo* in grass - white clover silage artificially contaminated with PR or PP, a microsilo experiment was performed. Mean silo density was 188 kg dry matter (DM) per m^3. After an ensiled period of 56 days, samples were taken for enumeration of *P. roqueforti s.l.* and for determination of DM content and pH. These results are presented in Table 5.

P. roqueforti s.l. counts were not significantly influenced by additive application, but evidently artificial contamination had a significant influence: PP contaminated silage contained significantly more *P. roqueforti s.l.* propagules than non-contaminated silage.

For both DM and pH at desiling, a significant interaction between the factors contamination and additive was detected. The DM content of silage without additive was significantly lowered upon *P. roqueforti s.l.* contamination. Application of Bv cell suspension resulted in significantly lower DM content of PR contaminated silage compared to PP contaminated silage. Additive application had a significant effect on PR contaminated silage: no additive resulted in a significantly higher DM content compared to Bv cell suspension. Silage pH without additive application was significantly lowered by PP contamination compared to non-contaminated and PR contaminated silage. No significant effect of contamination on pH was detected upon application of Bv cell suspension. In PR contaminated silage, treatment with Bv cell suspension resulted in a significantly reduced pH compared to no additive. Since no literature data could be found reporting the effect of *B. velezensis* on silage fermentation characteristics, the explanation for the observed differences in DM content and pH between objects remains elusive.

DISCUSSION

It can be concluded from the *in vitro* experiment that both Bv supernatant and cell suspension had an inhibiting effect on *P. roqueforti s.l.* growth registered as OD_{620}. Chitarra *et al.* (2003) demonstrated that Bv culture supernatant had a negative effect on *P. roqueforti s.s.* conidiospore germination. The currently described *in vitro* experiment generally confirms this finding, but ascribes an even more potent inhibition of conidiospore germination to Bv cell suspension. The presence of either Bv supernatant or Bv cell suspension did not trigger an increased ROC production by *P. roqueforti s.l.*, which is crucial for a candidate silage additive.

Based on the results of the *in vitro* experiment, *B. velezensis* appeared to be a promising antagonist towards *P. roqueforti s.s.* as well as *P. paneum*. Bv growth in CSI was however very limited despite aerobic incubation conditions. This can be due to the intrinsic nature of the species, but also to the fact that the strain was not adapted to acidic conditions prior to the experiment. To be able to successfully apply *B. velezensis* as a silage inoculant producing antifungal lipopeptides *in vivo* in silages, good bacterial growth in silage conditions is a prerequisite. *In vitro* in CSI, however, Bv growth was very poor. *B. velezensis* is an aerobic micro-organism (Liu *et al.* 2010, Ruiz-Garcia *et al.* 2005). Ruiz-Garcia *et al.* (2005) have found that *B. velezensis* can grow in the pH range of 5-10. For the *in vitro* experiment, CSI at pH 3.79 was used. This pH is well below pH 5, so the lack of Bv growth observed is very likely due to a too acidic growth medium. Velmurugan *et al.* (2009) have found that antifungal activity of *B. velezensis* remained stable in a pH range of 2-10 at 25 °C for 24 hours, but this was tested on culture supernatant and not on living bacteria. Taking the clear effect of Bv on conidiospore survival and germination *in vitro* into account, lipopeptides can be assumed to have been present in the culture media - most likely introduced by the Bv supernatant or cell suspension at the start of the experiment. Monitoring of lipopeptide production should definitely be included in future experiments. Adding lipopeptides as a silage additive instead of a *Bacillus*-based inoculant would also be an option, prone to future research.

The Bv cell suspension applied to the *P. roqueforti s.l.* contaminated grass - white clover silage was not capable of living up to the great expectations which had arisen in the *in vitro* experiment: no significant reduction of *P. roqueforti s.l.* numbers compared to no additive application could be detected in silage. Most likely, this is due to a very short aerobic phase of the ensiling process in microsilos and a quick pH-drop, which are both beneficial for silage quality. Another option is that the manufacturing process of the Bv cell suspension was not optimal since it was no commercially available product resulting from extensive R&D. Moreover, since *Bacillus* species can carry antibiotic resistance genes (Bernhard *et al.* 1978, Steinmetz and Richter 1994), it is of the outmost importance to select strains not bearing these genes as potential silage inoculant candidates. The *B. velezensis* strain NRRL B-23189 has not been checked for the absence of antibiotic resistance genes.

In conclusion, the applied Bv cell suspension was unsuccessful in displaying antifungal properties towards *P. roqueforti s.l.* in the context of the *in vivo* microsilo experiment. However, it must be pointed out that the microsilos used for the *in vivo* experiment mimic silage fermentation conditions well, but the feed-out phase is not simulated (Wambacq 2017). During feed-out, aerobic metabolism re-flourishes, allowing yeasts and fungi (e.g. *P. roqueforti s.l.*) but also *Bacilli* to proliferate. *B. velezensis* enumeration as well as quantification of lipopeptides at different time points during the different phases of the ensiling process would definitely provide more information about the antagonistic potential of *B. velezensis* in a silage matrix.

It would be promising if a *B. velezensis* based silage inoculant could survive the ensiling process, producing antifungal lipopeptides *in vivo* during feed-out of silage. This would be an elegant strategy to prevent mycotoxin production by toxigenic fungi since multiple mycotoxin remediation strategies (e.g. mycotoxin binders, microbial degradation) are available when prevention has failed, but their *in vivo* efficacy is highly questionable. Prevention efforts are definitely preferable (Avantaggiato *et al.* 2005, Awad *et al.* 2010, De Mil *et al.* 2015, Devreese 2013, Wambacq *et al.* 2016).

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Auerbach, H., Oldenburg, E. and Weissbach, F. (1998) Incidence of *Penicillium roqueforti* and roquefortine C in silages. *J Sci Food Agric* **76**, 565-572.

Avantaggiato, G., Solfrizzo, M. and Visconti, A. (2005) Recent advances on the use of adsorbent materials for detoxification of *Fusarium* mycotoxins. *Food Addit Contam* **22**, 379-388.

Awad, W. A., Ghareeb, K., Böhm, J. and Zentek, J. (2010) Decontamination and detoxification strategies for the *Fusarium* mycotoxin deoxynivalenol in animal feed and the effectiveness of microbial biodegradation. *Food Addit Contam* **27**, 510-520.

Bernhard, K., Schrempf, H. and Goebel, W. (1978) Bacteriocin and antibiotic resistance plasmids in *Bacillus cereus* and *Bacillus subtilis*. *J Bacteriol* **133**, 897-903.

Boichenko, D., Zelenkova, N., Vinokurova, N. and Baskunov, B. (2002) Factors contributing to roquefortine yield variability during cultivation of *Penicillium roquefortii*. *Appl Biochem Biotechnol* **38**, 32-35.

Boudergue, C., Burel, C., Dragacci, S., Favrot, M. C., Fremy, J., Massimi, C., Prigent, P., Debongnie, P., Pussemier, L. and Boudra, H. 2009. *Review of mycotoxin-detoxifying agents used as feed additives: mode of action, efficacy and feed/food safety*. Scientific report submitted to EFSA.

Boysen, M. E., Jacobsson, K.-G. and Schnürer, J. (2000) Molecular identification of species from the *Penicillium roqueforti* group associated with spoiled animal feed. *Appl Environ Microbiol* **66**, 1523-1526.

Chitarra, G., Breeuwer, P., Nout, M., van Aelst, A., Rombouts, F. and Abee, T. (2003) An antifungal compound produced by *Bacillus subtilis* YM 10–20 inhibits germination of *Penicillium roqueforti* conidiospores. *J Appl Microbiol* **94**, 159-166.

Cleveland, T. E., Dowd, P. F., Desjardins, A. E., Bhatnagar, D. and Cotty, P. J. (2003) United States Department of Agriculture - Agricultural Research Service research on pre-harvest prevention of mycotoxins and mycotoxigenic fungi in US crops. *Pest Manag Sci* **59**, 629-642.

Codex Alimentarius Commission (2002). *Proposed draft code of practice for the prevention (reduction) of mycotoxin contamination in cereals, including annexes on ochratoxin A, zearalenone, fumonisins and tricothecenes. CX/FAC02/21.* Codex Committee on Food Additives and Contaminants, joint FAO/WHO Food Standards Programme.

Cotty, P. J. and Bhatnagar, D. (1994) Variability among atoxigenic *Aspergillus flavus* strains in ability to prevent aflatoxin contamination and production of aflatoxin biosynthetic pathway enzymes. *Appl Environ Microbiol* **60**, 2248-2251.

De Mil, T., Devreese, M., De Baere, S., Van Ranst, E., Eeckhout, M., De Backer, P. and Croubels, S. (2015) Characterization of 27 mycotoxin binders and the relation with *in vitro* zearalenone adsorption at a single concentration. *Toxins* **7**, 21-33.

Delmulle, B. (2009) *Investigation of mycotoxin production in water-damaged mouldy interiors in connection with the sick building syndrome.* PhD thesis, Ghent University.

Devreese, M. (2013) *Development of in vitro and in vivo models for testing the efficacy of mycotoxin detoxifying agents and their possible interaction with oral absorption of veterinary drugs.* PhD thesis, Ghent University.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 March 2018 doi:10.20944/preprints201801.0273.v2

Dolci, P., Tabacco, E., Cocolin, L. and Borreani, G. (2011) Microbial dynamics during aerobic exposure of corn silage stored under oxygen barrier or polyethylene films. *Appl Environ Microbiol* **21**, 7499-7507.

Dorner, J. and Lamb, M. (2006) Development and commercial use of Afla-guard®, an aflatoxin biocontrol agent. *Mycotoxin Res* **22**, 33-38.

Droby, S., Chalutz, E., Wilson, C. and Wisniewski, M. (1989) Characterization of the biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit. Can J Microbiol **35**, 794-800.

Dunière, L., Sindou, J., Chaucheyras-Durand, F., Chevallier, I. and Thévenot-Sergentet, D. (2013) Silage processing and strategies to prevent persistence of undesirable microorganisms. *Anim Feed Sci Technol* **182**, 1-15.

Garon, D., Richard, E., Sage, L., Bouchart, V., Pottier, D. and Lebailly, P. (2006) Mycoflora and multimycotoxin detection in corn silage: experimental study. *J Agric Food Chem* **54**, 3479-3484.

Gourama, H. and Bullerman, L. B. (1995) Antimycotic and antiaflatoxigenic effect of lactic acid bacteria: a review. *J Food Prot* **58**, 1275-1280.

Frisvad, J. and Samson, R. (2004) Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*: a guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. In Studies in Mycology 49, ed. Samson, R. and Frisvad, J. pp. 1-174. Utrecht: Centraalbureau voor Schimmelcultures.

Jijakli, M. H. and Lepoivre, P. (1998) Characterization of an exo-β-1, 3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. *Phytopathology* **88**, 335-343.

Jouany, J. P. (2007) Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. *Anim Feed Sci Technol* **137**, 342-362.

Kabak, B., Dobson, A. D. and Var, I. (2006) Strategies to prevent mycotoxin contamination of food and animal feed: a review. *Crit Rev Food Sci Nutr* **46**, 593-619.

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A. and Gobbetti, M. (2000) Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. *Appl Environ Microbiol* **66**, 4084-4090.

Liu, X., Ren, B., Chen, M., Wang, H., Kokare, C., Zhou, X., Wang, J., Dai, H., Song, F., Liu, M., Wang, J., Wang, S. and Zhang, L. (2010) Production and characterization of a group of bioemulsifiers from the marine *Bacillus velezensis* strain H3. *Appl Microbiol Biotechnol* **87**, 1881-1893.

Mansfield, M. and Kuldau, G. (2007) Microbiological and molecular determination of mycobiota in fresh and ensiled maize silage. *Mycologia* **99**, 269-278.

McDonald, P., Henderson, A. and Heron, S. (1991) *The biochemistry of silage, 2nd edition.* Lincoln: Chalcombe Publications.

Monbaliu, S., Van Poucke, C., Detavernier, C. l., Dumoulin, F., Van De Velde, M., Schoeters, E., Van Dyck, S., Averkieva, O., Van Peteghem, C. and De Saeger, S. (2010) Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-MS/MS method. *J Agric Food Chem* **58**, 66-71.

Munimbazi, C. and Bullerman, L. (1998) Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus*. *J Appl Microbiol* **84**, 959-968.

Nam, M., Park, M., Kim, H. and Yoo, S. (2009) Biological control of strawberry *Fusarium* wilt couased by *Fusarium oxysporum* f. sp. *fragariae* using *Bacillus velezensis* BS87 and RK1 formulation. *J Microbiol Biotechnol* **19**, 520-524.

Niderkorn, V. (2007) *Activités de biotransformation et de séquestration des fusariotoxines chez les bactéries fermentaires pour la détoxification des ensilages de maïs* PhD thesis, Université Blaise-Pascal.

Nout, M., Bouwmeester, H., Haaksma, J. and Van Dijk, H. (1993) Fungal growth in silages of sugarbeet press pulp and maize. *J Agric Sci* **121**, 323-326.

O'Brien, M., Egan, D., O'Kiely, P., Forristal, P. D., Doohan, F. and Fuller, H. (2008) Morphological and molecular characterization of *Penicillium roqueforti* and *P. paneum* isolated from baled grass silage. *Mycol Res* **112**, 921-932.

O'Brien, M., O'Kiely, P., Forristal, P. and Fuller, H. (2007) Visible fungal growth on baled silage during the winter feeding season in Ireland and silage characteristics associated with the occurrence of fungi. *Anim Feed Sci Technol* **139**, 234-256.

O’Brien, M., O’Kiely, P., Forristal, P. D. and Fuller, H. T. (2007) Quantification and identification of fungal propagules in well-managed baled grass silage and in normal on-farm produced bales. *Anim Feed Sci Technol* **132**, 283-297.

Ohmomo, S., Tanaka, O. and Kitamoto, K. H. (1993) Analysis of organic acids in silage by high-performance liquid chromatography. *Bull Nat Grassl Res Inst* **48**, 51-56.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 March 2018 doi:10.20944/preprints201801.0273.v2

Ongena, M. and Jacques, P. (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol* **16**, 115-125.

Oude Elferink, S., Driehuis, F. and Gottschal, J. C. (2000) Silage fermentation processes and their manipulation. FAO Electronic Conference on Tropical Silage, Rome, pp. 17-30.

Petersson, S. and Schnürer, J. (1995) Biocontrol of mold growth in high-moisture wheat stored under airtight conditions by *Pichia anomala*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **61**, 1027-1032.

Pusey, P. L. (1989) Use of *Bacillus subtilis* and related organisms as biofungicides. *Pest Sci* **27**, 133-140.

Richard, D., Schiavi, B. and Joullie, M. (2004) Synthetic studies of roquefortine C: synthesis of isoroquefortine C and a heterocycle. *PNAS* **101**, 11971-11976.

Richard, E., Heutte, N., Sage, L., Pottier, D., Bouchart, V., Lebailly, P. and Garon, D. (2007) Toxigenic fungi and mycotoxins in mature corn silage. *Food Chem Toxicol* **45**, 2420-2425.

Romero, D., de Vincente, A., Rakotoaly, R., Dufour, S., Veening, J.-W., Arrebola, E., Cazorla, F., Kuipers, O., Paquot, M. and Perez-Garcia, A. (2007) The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. *Mol Plant Microbe Interact* **20**, 430-440.

Ruiz-Garcia, C., Bejar, V., Martinze-Checa, F., Llamas, I. and Quesada, E. (2005) *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Vélez in malaga, southern Spain. *Int J System Evolul Microbiol* **55**, 191-195.

Samson, R., Hoekstra, E. S., Frisvad, J. C. and Filtenborg, O. (2002) *Introduction to food-and airborne fungi, 6th edition.* Utrecht: Centraalbureau voor Schimmelcultures.

Schnürer, J. and Magnusson, J. (2005) Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sci Technology* **16**, 70-78.

Steinmetz, M. and Richter, R. (1994) Plasmids designed to alter the antibiotic resistance expressed by insertion mutation in *Bacillus subtilis*, through *in vivo* recombinatio. *Genetics* **142**, 79-83.

Ström, K., Sjögren, J., Broberg, A. and Schnürer, J. (2002) *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Appl Environ Microbiol* **68**, 4322-4327.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 March 2018 doi:10.20944/preprints201801.0273.v2

Velmurugan, N., Choi, M., Han, S. and Lee, Y. (2009) Evaluation of antagonistic activities of *Bacillus subtilis* and *Bacillus licheniformis* against wood-staining fungi: *in vitro* and *in vivo* experiments. *J Microbiol* **47**, 385-392.

Wambacq, E. (2017) *Penicillium roqueforti s.l.: growth and roquefortine C production in silages.* PhD thesis, Ghent University.

Wambacq, E., Vanhoutte, I., Audenaert, K., De Gelder, L. and Haesaert, G. (2016) Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: a review. *J Sci Food Agric* **96**, 2284-2302.

Wilkinson, J. and Davies, D. (2012) The aerobic stability of silage: key findings and recent developments. *Grass For Sci* **68**, 1-19.

Wilkinson, J. M. (2005) *Silage.* London: Chalcombe Publications.

Yiannikouris, A. and Jouany, J.-P. (2002) Mycotoxins in feeds and their fate in animals: a review. *Anim Res* **51**, 81-99.

Zuber, P., Nakano, M. and Marahiel, M. (1993) Peptide antibiotics. In: Sonenshein, A., Hoch, J. & Losick, R. (eds.) *Bacillus subtilis and other Gram-positive bacteria: biochemistry, physiology and molecular genetics.* Washington DC: American Society for Microbiology.

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Table 1 Gradient elution program for quantification of roquefortine C by LC-MS/MS

Time (min)	% mobile phase A	% mobile phase B
0-6	95	5
6-10	35	65
10-11	1	99
11-14	95	5
14-15	25	75
15-16	1	99
16-18	95	5
18-19	25	75
19-20	1	99
20-29	95	5

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Table 2 *In vitro* experiment evaluating the antagonistic effect of *B. velezensis* NRRL B-23189 (Bv) towards *P. roqueforti s.s.* MUCL 46746 (PR) and *P. paneum* CBS 112295 (PP): fungal growth registered as optical density at 620 nm (OD_{620}) after five days of incubation. Mean values are presented with their standard deviation between brackets. The effect of culture medium (CM) is indicated per fungal isolate by letter code.

Culture medium	PR growth		PP growth	
	mean (st.d.)	*CM*	mean (st.d.)	*CM*
100% CSI[*]	0.731 (0.047)	*abc*	0.819 (0.052)	*ab*
90% CSI + 10% sterile BHI[†]	0.689 (0.055)	*abcd*	0.738 (0.049)	*abc*
+ 10% Bv supernatant	0.553 (0.037)	*cd*	0.525 (0.021)	*e*
+ 10% Bv cell suspension	0.516 (0.069)	*d*	0.534 (0.017)	*de*
75% CSI + 25% sterile BHI	0.878(0.151)	*a*	0.726 (0.110)	*abcd*
+ 25% Bv supernatant	0.600 (0.093)	*bcd*	0.488 (0.045)	*e*
+ 25% Bv cell suspension	0.551 (0.074)	*cd*	0.466 (0.100)	*cde*
50% CSI + 50% sterile BHI	0.770 (0.067)	*ab*	0.845 (0.070)	*a*
+ 50% Bv supernatant	0.592 (0.066)	*bcd*	0.500 (0.056)	*e*

[*] CSI: corn silage infusion, [†] BHI: brain-heart infusion broth

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Table 3 *In vitro* experiment evaluating the antagonistic effect of *B. velezensis* NRRL B-23189 (Bv) towards *P. roqueforti s.s.* MUCL 46746 (PR) and *P. paneum* CBS 112295 (PP): conidiospore germination (%) after 24 hours of incubation. Mean values are presented with their standard deviation between brackets. The effect of culture medium (CM) is indicated per fungal isolate by letter code.

Culture medium	PR conidiospore germination mean (st.d.)	*CM*	PP conidiospore germination mean (st.d.)	*CM*
100% CSI*	40 (2)	*a*	37 (3)	*a*
90% CSI + 10% sterile BHI†	23 (5)	*bc*	28 (4)	*ab*
+ 10% Bv supernatant	34 (9)	*ab*	35 (7)	*a*
+ 10% Bv cell suspension	12 (2)	*d*	12 (9)	*de*
75% CSI + 25% sterile BHI	33 (3)	*ab*	23 (4)	*bc*
+ 25% Bv supernatant	17 (5)	*cd*	16 (4)	*cd*
+ 25% Bv cell suspension	15 (5)	*cd*	6 (3)	*e*
50% CSI + 50% sterile BHI	18 (4)	*cd*	19 (3)	*bcd*
+ 50% Bv supernatant	14 (2)	*cd*	13 (3)	*de*

* CSI: corn silage infusion, † BHI: brain-heart infusion

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Table 4 *In vitro* experiment evaluating the antagonistic activity of *Bacillus velezensis* NRRL B-23189 (Bv) towards *P. roqueforti s.s.* MUCL 46746 (PR) and *P. paneum* CBS 112295 (PP): screening of roquefortine C production (µg g^{-1} freeze-dried fungal biomass) after five days of incubation.

Culture medium	PR	PP
100% CSI*	4.68	1.18
90% CSI + 10% sterile BHI†	5.91	1.29
+ 10% Bv supernatant	6.80	1.22
+ 10% Bv cell suspension	5.46	1.71
75% CSI + 25% sterile BHI	10.92	2.72
+ 25% Bv supernatant	3.48	1.03
+ 25% Bv cell suspension	3.93	2.44
50% CSI + 50% sterile BHI	4.13	1.32
+ 50% Bv supernatant	2.51	1.48

* CSI: corn silage infusion, † BHI: brain-heart infusion

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Table 5 Microsilo experiment with perennial ryegrass-white clover, artificially contaminated with *P. roqueforti s.s.* MUCL 46746 (PR) and *P. paneum* CBS 112295 (PP), to evaluate the antagonistic effect of *B. velezensis* NRRL B-23189 (Bv) cell suspension: *Penicillium roqueforti s.l.* counts, dry matter content and pH at desiling after 56 days. Mean values are presented with their standard deviation between brackets. Statistically significant effects of fungal contamination (FC) and additive (Add.) are indicated by letter codes. Significant interaction between these two factors is indicated by °-symbols, and the effect of one factor is determined per level of the other factor (designated by lettercodes with and without ‘-symbols).

OBJECTS		*P. roqueforti s.l.* (log_{10} spores g^{-1} FM)		Dry matter at desiling ($g\ kg^{-1}$ FM)			pH at desiling		
FC.	Add.	mean (st.d.)	*FC.*	mean (st.d.)	*FC.°*	*Add.°*	mean (st.d.)	*FC.°*	*Add.°*
No	No	< 1.69	*a*	431 (2)	*a*	-	4.83 (0.05)	*a*	-
PR	No	2.08 (0.21)	*ab*	413 (0)	*b*	*a*	4.84 (0.04)	*a*	*a*
	Bv	1.79 (0.36)		377 (5)	*b’*	*b*	4.53 (0.06)	*a’*	*b*
PP	No	2.40 (0.19)	*b*	397 (3)	*c*	*a’*	4.62 (0.07)	*b*	*a’*
	Bv	2.32 (0.14)		402 (5)	*a’*	*a’*	4.53 (0.08)	*a’*	*a’*

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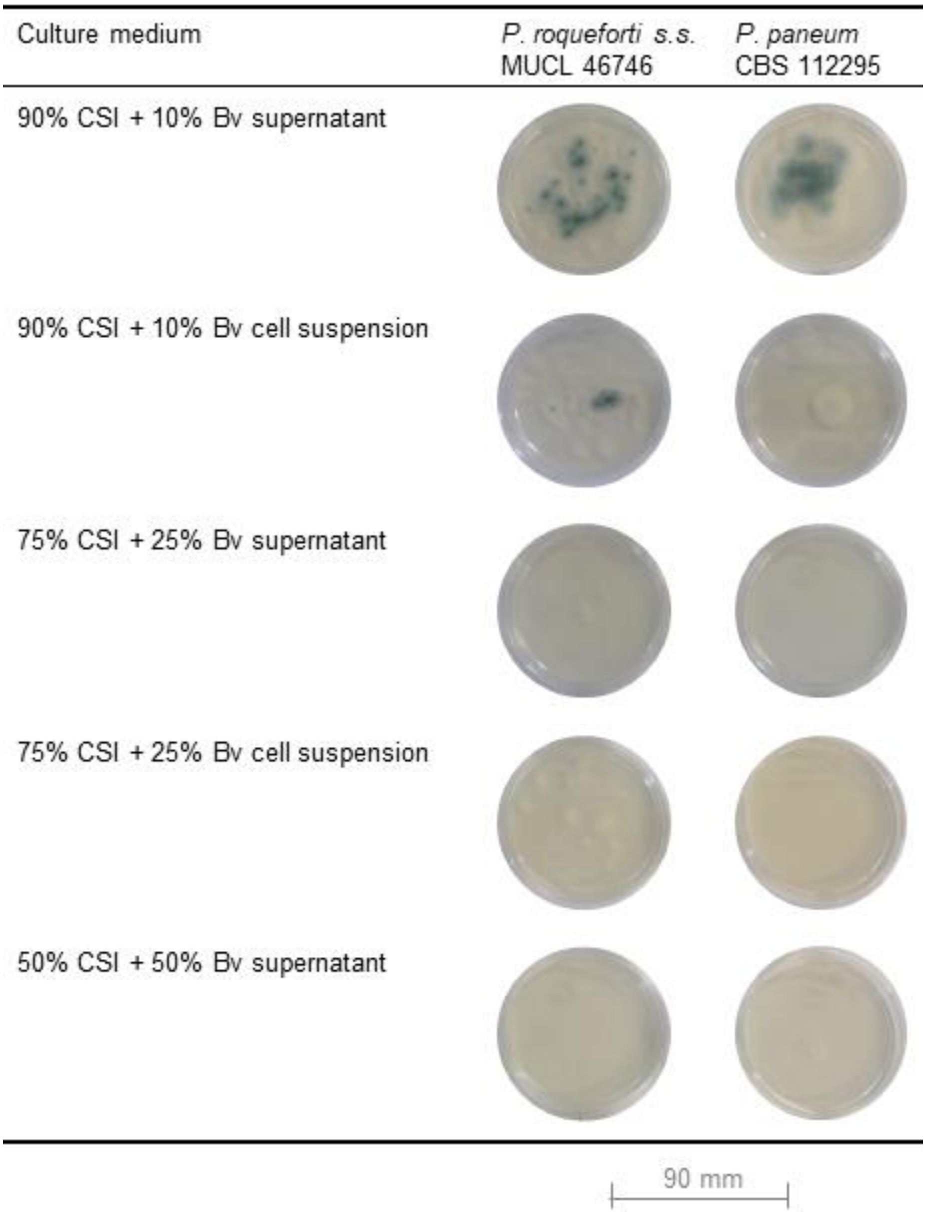

Fig. 1 *In vitro* experiment evaluating the antagonistic effect of *B. velezensis* NRRL B-23189 (Bv) towards *P. roqueforti s.s*. MUCL 46746 and *P. paneum* CBS 112295: streak-plates to monitor conidiospore survival after 24 hours of incubation.