Received: 4 October 2017 | Accepted: 13 February 2018

DOI: 10.1111/jph.12700

ORIGINAL ARTICLE

WILEY Journal of Phytopathology

# Biocontrol of strawberry fruit infected by *Botrytis cinerea*: Effects on the microbial communities on fruit assessed by next-generation sequencing

Andre Freire Cruz[1] | Geleta Dugassa Barka[2] | Justine Sylla[3] | Annette Reineke[3]

[1]Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan

[2]Laboratório de Biotecnologia do Cafeeiro, Universidade Federal de Viçosa, Viçosa, Brazil

[3]Department of Crop Protection, Geisenheim University, Geisenheim, Germany

**Correspondence**
A. F. Cruz, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan.
Email: andre@kpu.ac.jp

**Funding information**
Svenska Forskningsrådet Formas; Alexander von Humboldt-Foundation - AvH (Germany); Federal Ministry of Food, Agriculture and Consumer Protection —BMELV (Germany)

## Abstract

Fruit grey mould, caused by the fungus *Botrytis cinerea*, is known to be a harmful disease of strawberry at postharvest stage. However, effects of an application of biological control agents (BCAs) on strawberry fruit in terms of shift in the microbial community are still unknown. The present research aimed to investigate the effects of an application of BCAs on postharvest microbial populations present on strawberry fruits. Strawberry plants were sprayed with three kinds of BCA, RhizoVital 42 fl. (*Bacillus amyloliquefaciens* FZB42), Trianum-P (*Trichoderma harzianum* T22) and Naturalis (*Beauveria bassiana* ATCC 74040), targeting *Botrytis cinerea* fungus. Control plots were composed of water and fungicide treatments. Microbial communities (bacteria and fungi) were analysed via next-generation sequencing on an Illumina MiSeq. Analysis of 16S RNA and ITS rRNA sequences indicated that the BCAs application modified both bacterial and fungal community compositions and diversity. An application of two BCAs together had more effects on microbial community composition than a single application. These results suggest that BCAs can modify bacterial and fungal community composition and diversity on strawberry fruits, which may consequently improve the efficiency and establishment of these products on control of postharvest diseases of fruits, such as grey mould.

KEYWORDS

bacteria, biological control, fungi, grey mould, illumina, postharvest

## 1 | INTRODUCTION

Strawberry (*Fragaria ananassa* Duchesne) is a high-value cash crop, and its production has been steadily increasing worldwide (Essghaier et al., 2009). As strawberry fruit is soft and perishable, it is prone to mechanical injuries, desiccation, decay and adverse physiological conditions during harvest or storage (Bhaskara Reddy, Belkacemi, Corcuff, Castaigne, & Arul, 2000). Likewise, its susceptibility to diverse phytopathogens, including fungi, bacteria and viruses, has limited its yield (Schestibratov & Dolgov, 2005). Botrytis fruit rot, also referred to as "grey mould", caused by the ubiquitous fungus *Botrytis cinerea*, is a harmful disease of strawberry, infecting flowers and mature fruit both in the field and at the postharvest stage. Infections by *B. cinerea* cause serious crop losses in strawberry cultivation worldwide. Studies in Tunisia have reported a yield loss of 25% if no treatment is applied (Essghaier et al., 2009). In Florida State, USA, even in well-managed fields, the losses from fruit rot caused by this fungus may exceed 50% when environmental conditions, such as humidity and temperature, are favourable to disease development (Vorotnikova, VanSickle, & Borisova, 2012).

In the recent past, the frequent use of chemical fungicides in strawberry cultivation has resulted in the evolution of fungicide-resistant *B. cinerea* isolates in the field (Freeman et al., 2004; Leroch et al., 2013). Moreover, due to rising health and environmental

  

concerns about the intensive use of chemical pesticides, control methods targeting strawberry grey mould increasingly consider alternative strategies, including the use of biological control agents (BCAs). The first BCA commercially applied to control strawberry grey mould in glasshouse production systems was the antagonistic fungus *Trichoderma harzianum*, isolate T-39 (Tronsmo & Dennis, 1977). In addition, the antagonistic bacterium *Bacillus subtilis*, isolate QST713, is widely considered to be one of the most effective BCAs in controlling plant diseases, including grey mould (Wei, Hu, & Xu, 2016). Both these BCAs have been reported as successful in controlling *B. cinerea* in strawberry (Essghaier et al., 2009; Freeman et al., 2004). However, the introduction of BCAs to the plant microbiome and the colonization of the plant's phyllosphere might cause shifts in the microbial community, as organisms compete for resources or are affected by toxic components produced by one of the players in this system (Wei et al., 2016; Xu, Jeffries, Pautasso, & Jeger, 2011). In addition to the potential effects on the composition of the microbial community present in the phyllosphere, the introduction of an alien microbe could result in shifts, for example in metabolite composition or in symbiotic interactions between the host plant and other microbial inhabitants (Knief, 2014; Rout, 2014).

Accordingly, an analysis of the putative effects of introducing BCAs into the plant's phyllosphere is of vital interest in evaluating BCAs in biocontrol programs, both for their effects directly on the target organisms and indirectly on other microorganisms present. In this regard, previous studies by Sylla et al. (2013), Sylla, Alsanius, Krüger, and Wohanka (2015); Hunter, Hand, Pink, Whipps, and Bending (2010) recently assessed the effects of applying BCAs on the microbial communities of strawberry leaves infected with *B. cinerea*, using both culture-dependent and independent methods. These studies detected no significant effect arising from applying a BCA on microbial populations (bacteria and fungi) present on strawberry leaves by a culture-dependent approach. However, a reduced diversity of fungal communities was evident, caused by competitive displacement shortly after BCA introduction, according to the pyrosequencing data (Sylla et al., 2013).

Here, we applied next-generation sequencing (NGS) using the Illumina platform to analyse the effects of applying different BCAs on fungal and bacterial communities present on strawberry fruits infected with *B. cinerea*. We used strawberry fruit samples from the same experiment that had previously assessed effects of BCAs on composition of microbial leaf communities (Sylla et al., 2013).

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental field

Fruit samples were taken from a strawberry field experiment located at Geisenheim University, Germany. For a detailed description, see Sylla et al., 2013. Briefly, a field was cultivated with strawberry (*Fragaria x ananassa* Dutch. "Elsanta") plants, without any measures to control pests and/or diseases. Three commercially available BCAs for *B. cinerea* were applied to the plants: namely, a product based on *Bacillus amyloliquefaciens* FZB42 at $2.5 \times 10^{10}$ endospores/ml [RhizoVital 42 fl. ("Rhizo"), ABiTEP GmbH], *T. harzianum* T22 at $1 \times 10^{9}$ conidia/g [Trianum-P ("Boni"), Koppert Biological Systems] and the entomopathogenic fungus *Beauveria bassiana* strain ATCC 74040 [Naturalis® ("Natura"), CBC (Europe) S.r.l., Italy]. These products were applied six times in the year 2010 according to the manufacturer's instructions, alone or in combinations ("Rhizo-Boni", "Rhizo-Natura", "Boni-Natura" and "Rhizo-Boni-Natura"). The control plots were treated with water ("Cont") or synthetic fungicides (Signum, Switch, Teldor and Ortiva: "Fungicide"). The treatments were arranged in four replications, and the whole experiment was conducted for 3 months. Fruits were harvested 1 week after the last application. Fifteen fruits per treatment were taken and transported to the laboratory in cooled boxes. They were homogenized with 250 ml of PBS buffer solution (NaCl 0.13 M, KCl 0.1 M, $Na_2HPO_4$ 10 mM, $KH_2PO_4$ 1.7 mM, pH 7.4) and submitted to sonification for 7 min, followed by centrifugation at 14,000× *g* for 20 min at 4°C. The cell pellets were resuspended with 40 ml of PBS, transferred to 50 ml centrifuge tubes and stored at −20°C. After the storage period, tubes were centrifuged at 5,000× *g* at 4°C for 20 min, and the bottom phase containing the fruit pieces was used for DNA extraction.

### 2.2 | DNA isolation and metagenome analysis

DNA was extracted from 0.3 g of cell pellet using the Power Soil DNA Isolation Kit (Sued-Laborbedarf GmbH, Gauting, Germany). DNA samples with a minimum concentration of ≥5 ng/µl were selected for further metagenome analysis. As a result, samples treated with Boni and Rhizo-Natura were maintained with four replicates, while for the other treatments, only three replicates were available. Fragments of the bacterial 16S rRNA genes were amplified using the universal primer pairs 534f-CS1 (5`-CCAGCAGCCGCGGTAAT-3`) and 783r-CS2 (5`-GGTCTACC MGGGTATCTAATCCKG-3`), which are reliable to estimate bacterial abundances on plants as they exclude the mitochondria and chloroplast genes (Rastogi, Tech, Coaker, & Leveau, 2010). Parts of the fungal ITS gene were amplified with the primers ITS1-CS1 (5`-CTTGGTCATTTAGAGGAAGTAA-3`) and 5.8S-CS2 (5`-GGTCTAACTTTYRRCAAYGGATCWCT-3`) (Buée et al., 2009). All primers contained their respective adapters, CS1 (ACACTGACGAC ATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTT) (Fluidigm Co., USA) to allow sequencing. PCR reaction mixtures consisted of 12.5 µl of KAPA HiFi HotStart Ready Mix (Kapa Biosystems Co., USA), 5 µl of 1 µM primers and 2.5 µl (equivalent to 12 ng) of genomic DNA in a final volume of 25 µl. The reaction conditions were as follows: predenaturation for 3 min at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, with a final extension of 5 min at 72°C. Library construction (index PCR) using the PCR products and specific barcodes and adapters (Fluidigm Co., USA), as well as the metagenome assay, was carried out by Illumina MiSeq at Genome Quebec Co., Canada. Bacterial 16S data were reassembled using Galaxy software (https://usegalaxy.org), and

the remaining bioinformatic analysis of the data was performed in the CloVR pipeline (http://clovr.org) (Angiuoli et al., 2011).) For fungal ITS data, the QIIME pipeline (http://qiime.org) (Caporaso et al., 2010) was applied. The USEARCH 6.1 was used for the chimera check. QIIME software was also used to assess the relative abundance of operational taxonomic units (OTUs), microbial diversity indexes (Shannon–Wiener, Chao and Simpson) and principal component analysis (PCA) on strawberry fruit. OTU clusters were defined by a 97% identity threshold, and the data sets were submitted to rarefaction analysis before diversity analysis. The quality filtering was applied at a Phred score ≥ Q20. The taxonomy base analysis was accessed by Ribosomal Database Project (RDP) classifier supplemented with BLAST and mothur. Differences between treatments for the relative abundance and diversity indexes were estimated by analysis of variance (ANOVA) after checking the normality distribution with the Shapiro–Wilk test. Moreover, one-way ANOSIM (analysis of similarities based on OTUs without singletons) was performed with IBM SPSS 20 statistical software. Based on nonparametrical tests (for 16S), and Bray–Curtis dissimilarity (for ITS), respectively, was used to evaluate the effects on fungal and bacterial community structure between the different BCA treatments applied alone and in combination. All the metagenome sequences were registered with the accession number DRA006624 in the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp).

## 3 | RESULTS

The total number of sequences obtained from all fruit samples was 4,167,795 (average number of reads—ANR 182,563) and 4,658,998 (207,034 ANR) for 16S rRNA and ITS rRNA, respectively. These reads were submitted to filtering based on a Phred quality score ≥20. Thus, 3,653,258 16S rRNA (87%) sequences were assigned to bacteria and 4,156,897 ITS rRNA (89%) to fungi. The following results were consistently displayed at class level because the number of reads was considerable to provide the required information at this taxonomic level.

### 3.1 | Effects of BCAs on bacterial communities on strawberry fruit

The bacterial relative abundance (RA) within the 16S rRNA profiles at class level indicated that strawberry fruit was mainly colonized

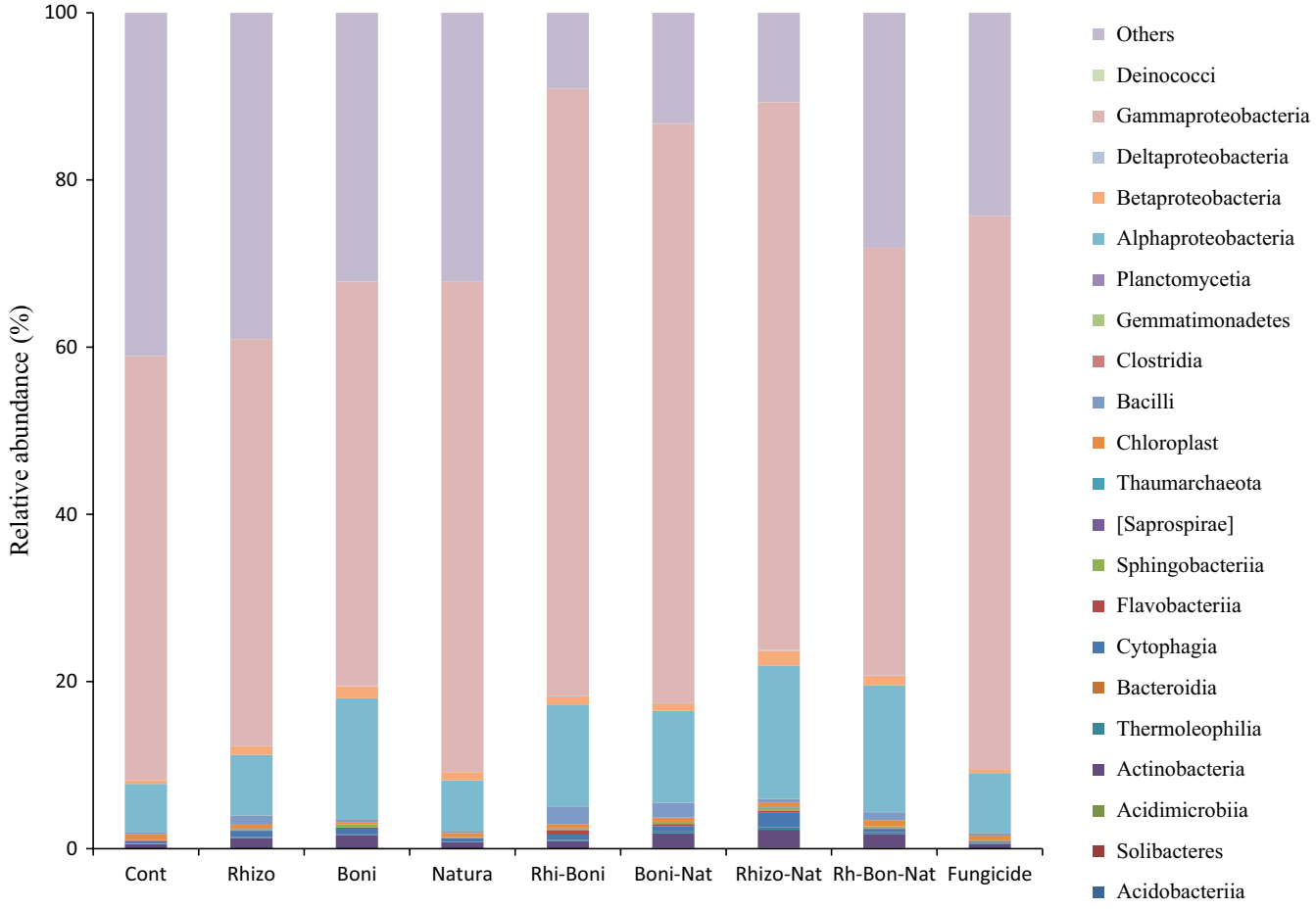


**FIGURE 1** Distribution of phylogenetical groups of bacteria on strawberry fruit treated with water as a control (Cont.l), fungicide and different biological control agents (Rhizo = RhizoVital 42 fl., Boni = Trianum-P, Natura (Nat) = Naturalis®) applied individually or in combinations

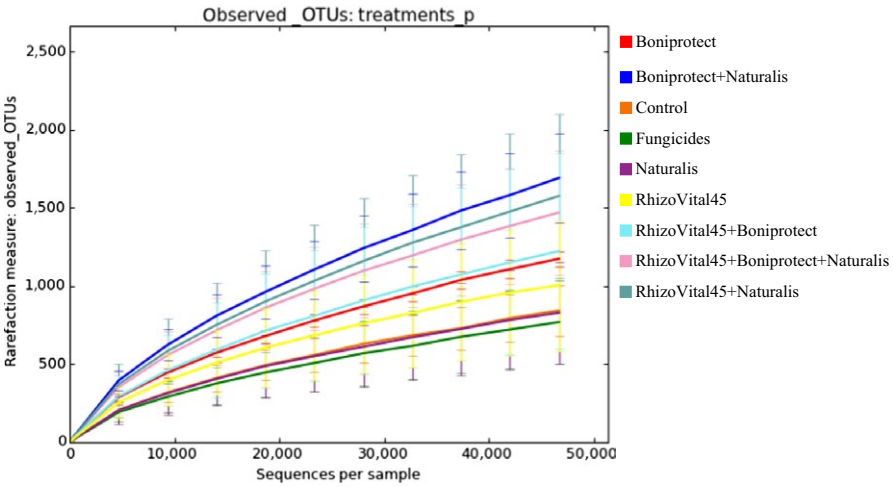


**FIGURE 2** Rarefaction curves indicating the observed number of OTUs within the 16S RNA gene sequences of strawberry fruit treated with water (Control), fungicide and different biological control agents (Rhizo = RhizoVital 42 fl., Boni = Trianum-P, Natura (Nat) = Naturalis®) applied individually or combined. Vertical bars represent standard error (*SE*) (*n* = 3)

by the *Alphaproteobacteria* and *Gammaproteobacteria*. The RA of these classes was larger in treatments with a double BCA application (Rhizo-Boni, Rhizo-Natura and Boni-Natura) and with a single Boni application. The RA of *Alphaproteobacteria* presented a similar pattern in the control, Natura and fungicide treatments. The fungicide treatments did not affect the composition of most bacterial classes compared to the control, except for *Alphaproteobacteria* and *Gammaproteobacteria*, which showed higher RA in fungicide treatments. In general, the majority of bacterial classes presented a higher RA in combined applications of BCAs compared to control and fungicide treatments. However, this difference was not found between single and triple treatments (Rhizo-Boni-Natura). The group of *Bacilli* was strongly present in Boni treatments and in those with Rhizo and Rhizo-Natura, whereas it was very low in control and fungicide-treated samples (Figure 1). Significant effects of treatments on abundance of bacterial and fungal classes are summarized in Table S1, indicating that abundance of most of the bacterial classes was affected by one of the treatments. Members of the

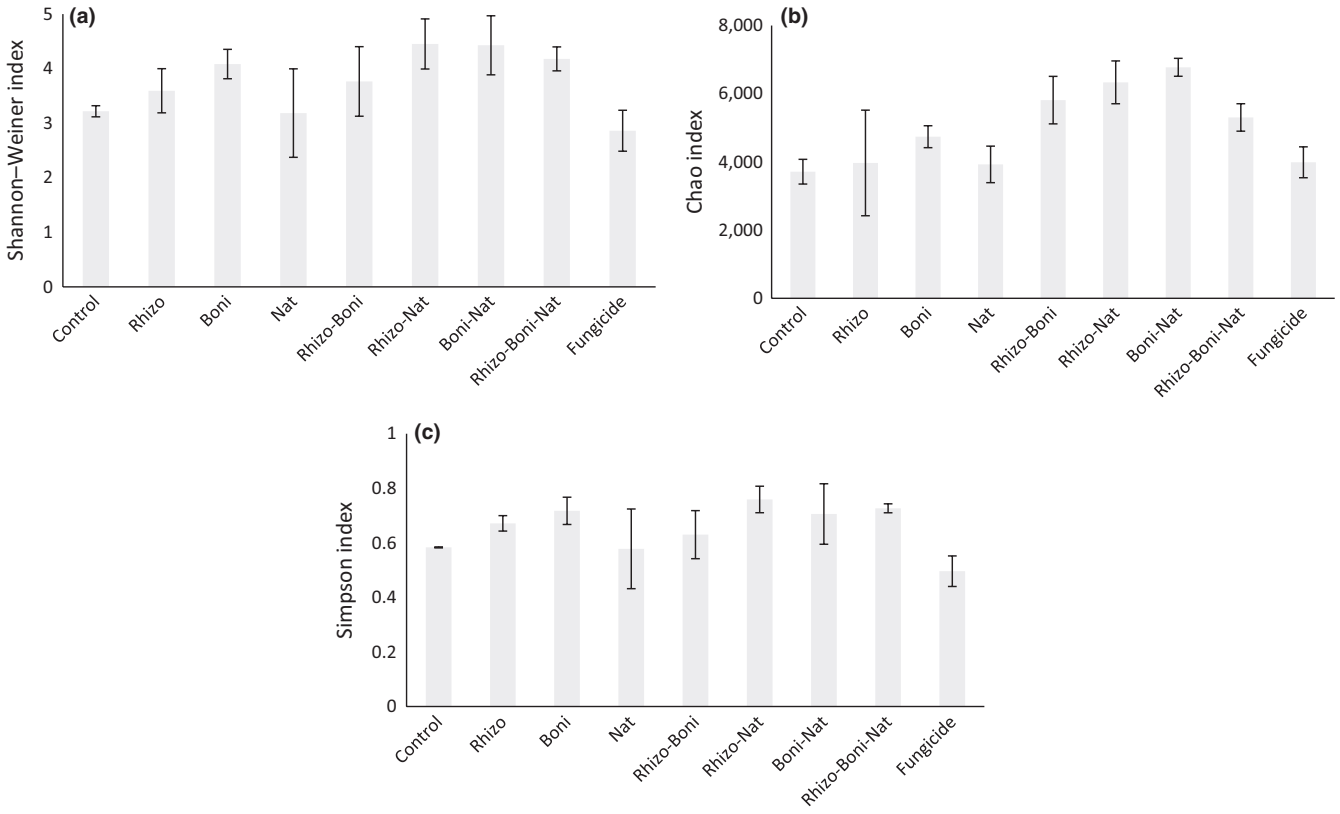


**FIGURE 3** Bacterial diversity according to Shannon–Wiener (a), Chao (b) and Simpson (c) index of strawberry fruit treated with water (Control), fungicide and different biological control agents (Rhizo = RhizoVital 42 fl., Boni = Trianum-P, Natura (Nat) = Naturalis®) applied individually or combined. Vertical bars represent standard error (*SE*) (*n* = 3)

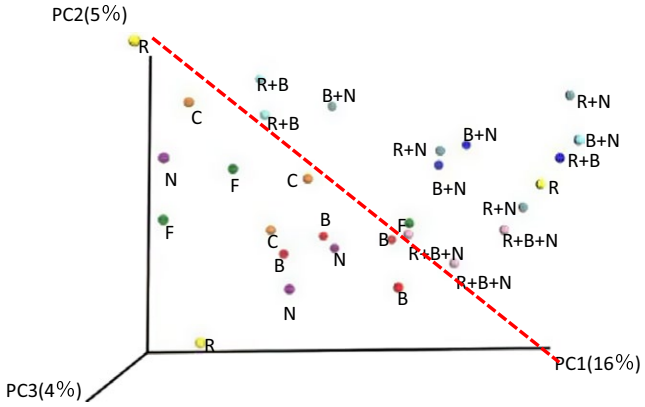


**FIGURE 4** Principal component analyses based on 16S rRNA gene sequences of strawberry fruit treated with water (Control), fungicide and different biological control agents applied individually or combined. C = control, R = RhizoVital 42, N = Naturalis®, B = Trianum-P, B+N = Trianum-P+Naturalis®, R+N = RhizoVital 42 + Naturalis®, R+B = RhizoVital 42 + Trianum-P, R+N = RhizoVital 42 + Naturalis®, R+B+N = RhizoVital 42 + Trianum-P+Naturalis®, F = Fungicide

Alphaproteobacteria and Gammaproteobacteria, which were among the most abundant classes present in all fruits samples, were significantly affected in their abundance by application of BCAs (Table S1).

To estimate the diversity at species level and to perform comparisons between treatments, all sequences were clustered into OTUs. After computing rarefaction curves for each treatment, more bacterial OTUs were observed on fruit treated with Boni-Natura and Rhizo-Natura compared to the other samples (Figure 2). The lowest number of OTUs was obtained on fruit treated with fungicides and those obtained from the control treatment. In addition, the diversity indexes represented by Shannon–Wiener, Chao and Simpson (Figure 3) demonstrated that plants with an application of two and three different BCAs, as well as single treatments with Boni, showed a more diverse bacterial community on strawberry fruits compared to treatments with fungicide or Naturalis. According to the ANOVA, only the Simpson index showed significant effects as a result of the different treatments (Table S1).

Principal component analysis (PCA) showed a clear grouping of samples according to their treatments, in particular a contrast can be noticed between the right part of the PCA composed of the combined BCA application (double and triple) and the left one with the

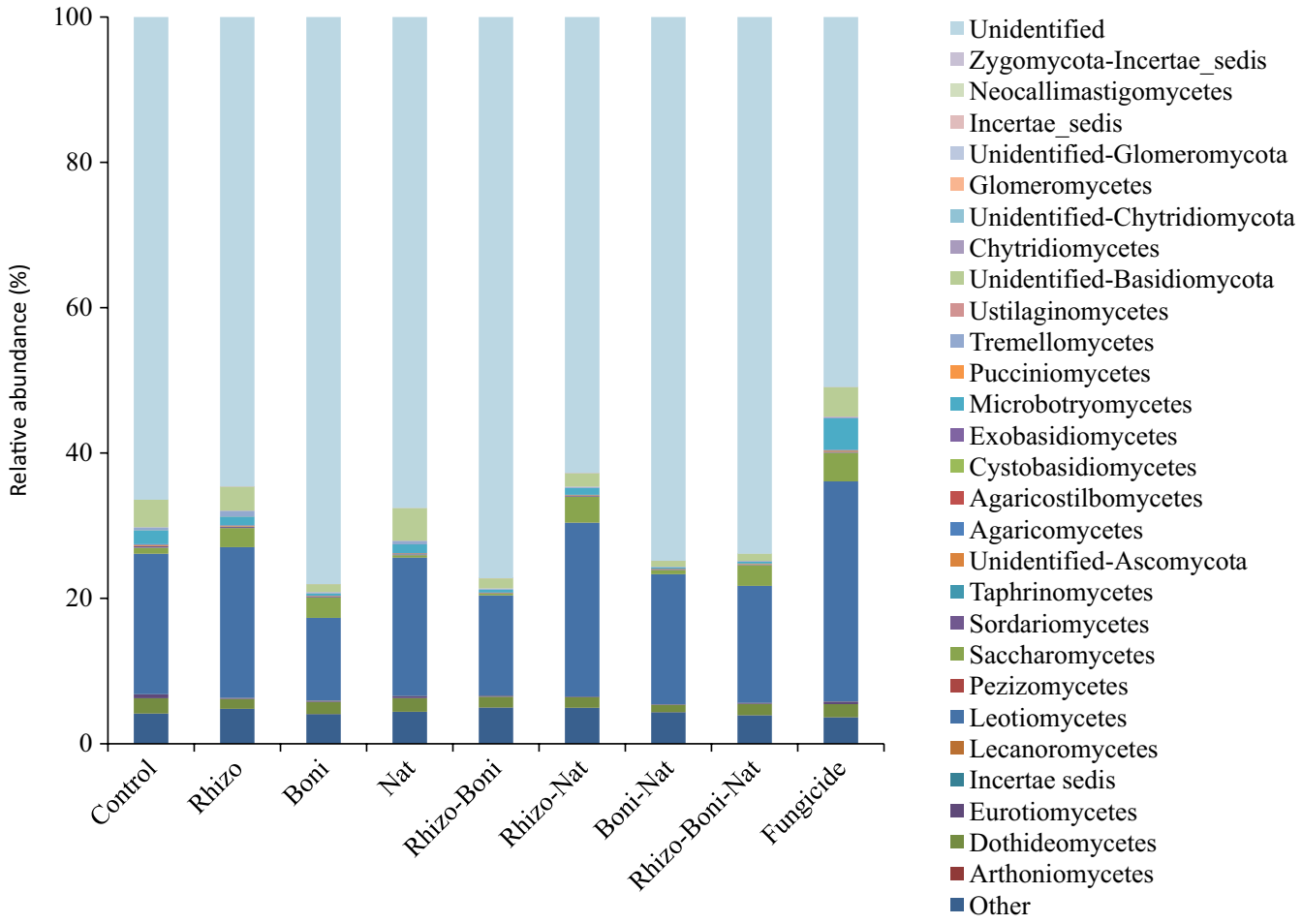


**FIGURE 5** Distribution of phylogenetical groups of fungi on strawberry fruit treated with water (Control), fungicides and different biological control agents (Rhizo = RhizoVital 42 fl., Boni = Trianum-P, Natura (Nat) = Naturalis®) applied individually or combined

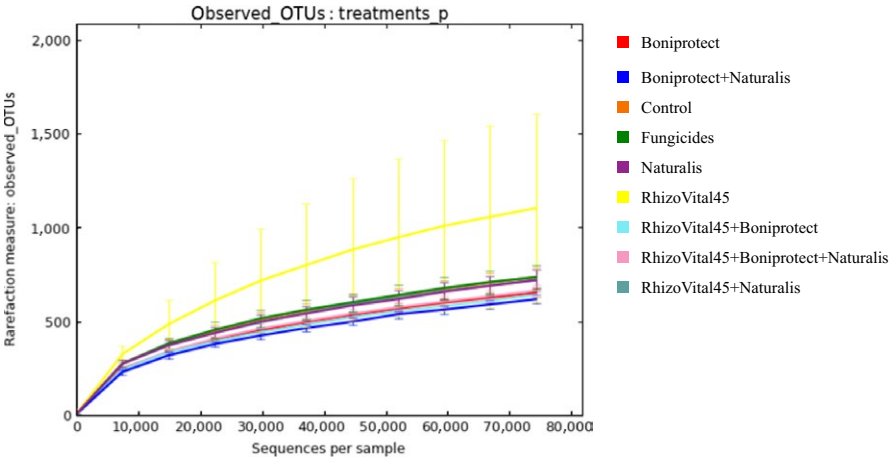


**FIGURE 6** Rarefaction curves indicating the observed number of OTUs within the ITS rRNA gene sequences of strawberry fruit treated with water (Control), fungicides and different biological control agents (Rhizo = RhizoVital 42 fl., Boni = Trianum-P, Natura (Nat) = Naturalis®) applied individually or combined. Vertical bars represent standard error (*SE*) ($n$ = 3)

control, fungicide and single application of BCAs. Within the left group, another contrast is found between the subgroup control and fungicide treatments and those samples treated with single applications of BCAs (Natura and Boni) (Figure 4). More detailed, the overall effect of treatments was observed, with special contrast between Natura and other treatments, and similar fact occurred to Rhizo-Boni-Nat (Table S2).

### 3.2 | Effects of BCAs on fungal communities on strawberries

The RA distribution across treatments showed that the Dothideomycetes and Leotiomycetes classes dominated the observed sequences, and the treatments mostly contained profiles distinguished depending on the treatment. Leotiomycetes abundance

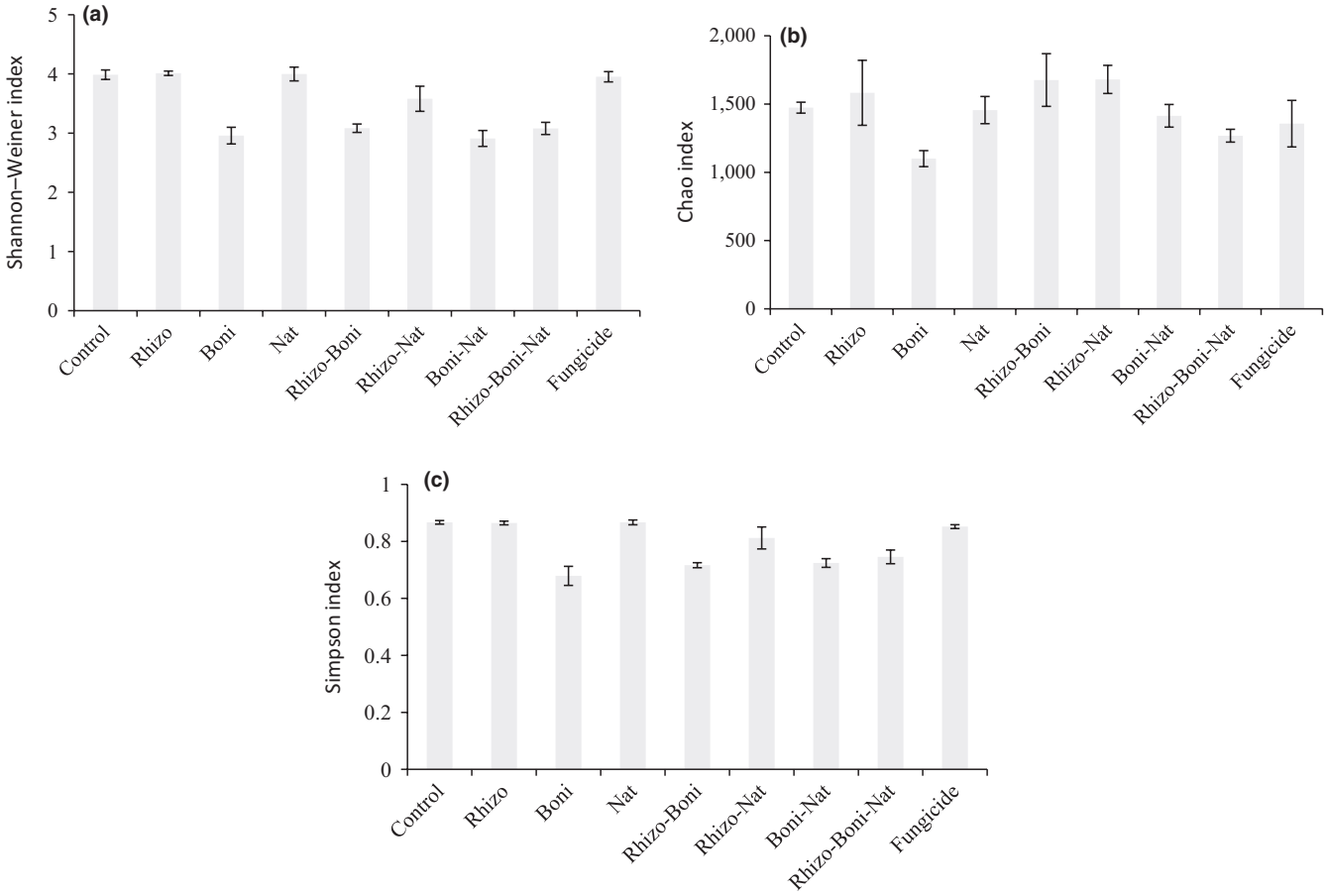


**FIGURE 7** Fungal diversity according to Shannon–Wiener (a), Chao (b) and Simpson (c) index of strawberry fruit treated with water (Control), fungicides and different biological control agents (Rhizo = RhizoVital 42 fl., Boni = Trianum-P, Natura (Nat) = Naturalis®) applied individually or combined. Vertical bars represent standard error (*SE*) ($n$ = 3)

was higher on fruits treated with fungicides and lower on fruits treated with Boni and Rhizo-Boni, respectively. Microbotryomycetes were more abundant on fruits treated with water (control) or fungicide. The Rhizo, Boni, Rhizo-Natura and fungicide treatments had greater RA of Saccharomycetes and Agaricostilbomycetes compared to other ones. Tremellomycetes were found mostly on fruits to which Rhizo had been applied (Figure 5). An ANOVA analysis revealed that Dothideomycetes and Sordariomycetes were strongly affected in their abundance by BCA application (Table S1).

A comparison of rarefaction curves illustrating the observed number of fungal OTUs present on strawberry fruits revealed that the highest number of OTUs was obtained on fruit treated with Rhizo compared to the other samples. In fact, there was a big standard error in this treatment caused by one sample, although the overall difference was not significant (Figure 6). An analysis of different diversity indices showed that all treatments containing Boni (Boni, Rhizo-Boni, Boni-Natura and Rhizo-Boni-Natura) resulted in fungal communities present on fruit with a lower diversity than the other treatments (Figure 7). This effect was most prominent for the Shannon (Figure 7a) and Chao indices (Figure 7b), while the Simpson index showed only slight differences in diversity of strawberry fruit fungal communities between samples (Figure 7c). In addition, the significant effect of BCAs on fungal diversity was found for all diversity indices (Table S1).

Similarly, a PCA analysis of strawberry fruit fungal communities separated the samples into two distinct groups (treatments with and without Boni), confirming the effects of an application of the product Boniprotect on the composition of the fungal community on strawberry fruits (Figure 8). Similar to the results for bacterial abundance, a significant effect of BCA treatments was detected, indicating a remarkable difference in between the water control and fungicide treatment compared to other treatments. (Table S2).

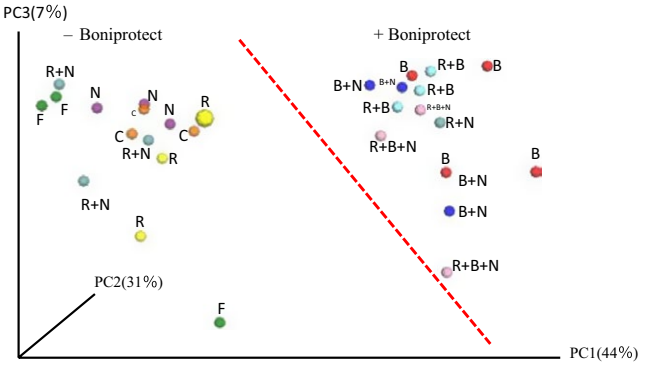


**FIGURE 8** Principal component analysis based on ITS rRNA gene sequences of strawberry fruit treated with water (Control), fungicide and different biological control agents applied individually or combined. C = control, R = RhizoVital 42, N = Naturalis®, B = Trianum-P, B+N = Trianum-P+Naturalis®, R+N = RhizoVital 42 + Naturalis®, R+B = RhizoVital 42 + Trianum-P, R+N = RhizoVital 42 + Naturalis®, R+B+N = RhizoVital 42 + Trianum-P+Naturalis®, F = Fungicide

## 4 | DISCUSSION

The results of the present study indicate that bacterial and fungal communities present on strawberry fruit show a distinct response to the application of BCAs. The diversity of bacterial and fungal communities varied according to the BCA applied. In strawberry, a characterization of microbial communities present on fruits can provide useful information and might assist for further recommendations to optimize BCA applications to control grey mould disease.

However, it is often difficult to discriminate between the synergetic and antagonistic effects of BCAs when it comes to *B. cinerea* control in strawberry (Freeman et al., 2004; Leroch et al., 2013). Any effect of BCAs on controlling grey mould diseases cannot be solely accessed by evaluating the composition of microbial communities on the respective fruits. Some reports showed that the abundance of bacterial communities was correlated with the efficacy of BCAs originating from different *Bacillus* species to control grey mould of strawberry (Berg, 2009; Wei et al., 2016) and anthracnose disease of papaya fruit (Hasan, Mahmud, Ding, & Kadir, 2013). In another scenario (Sylla et al., 2013), the effect of Boni on fungal composition and diversity in strawberry leaves was also reported.

The high relative abundance of the Bacilli class could be an indicator of the efficacy of combined BCA application in controlling strawberry grey mould (*B. cinerea*). BCA-treated samples showed a significant increase in the number of bacterial classes, while overall analysis shown by principal component analysis (PCA) indicated a strong contrast between BCA-treated fruit and the controls. This result is similar to earlier work, performed on strawberry leaves, in which there was no significant effect on the bacterial diversity at order level by treating with Rhizo, Boni or Natura as a single BCA application (Sylla et al., 2013). Moreover, the introduction of Boni resulted in significant changes in fungal diversity on strawberry fruits.

Operational taxonomic unit analysis indicated that an application of the bacillus product Rhizo induced more fungal diversity on strawberry fruits compared to any of the other treatments. The combination Rhizo-Boni-Natura or as a triple treatment combined with Boniprotect and Naturalis resulted in high OTU numbers. The effect of a double application of Rhizo-Natura did not significantly affect the phyllosphere diversity of fungi and bacteria at class level, when pyrosequencing was used in strawberry and hot pepper (Bhaskara Reddy et al., 2000; Kim, Cho, Jeong, Lee, & Kim, 2010).

In the present work, however, increased fungal biodiversity at class level was observed when Rhizo was combined with Natura. All Boni-treated groups showed less abundance of Leotiomycetes and Microbotryomycetes compared to control and fungicide treatments, suggesting an effect of this group of BCAs in suppressing classes containing *Botrytis* genera. High efficiency of the BCA product Trichodex containing *Trichoderma harzianum* on *B. cinerea* was reported, as well as entomopathogenic activity (Freeman et al., 2004). Of considerable importance is the high relative abundance

of Basidiomycota, indicating that it may have an inhibitory effect against *B. cinerea*, as was reported in other plants (Freeman et al., 2004; Xu, Robinson, Jeger, & Jeffries, 2010). Overall, PCA analysis showed the biggest contrast in all the Boni treatments and non-Boni samples, resulting in a clear distinction between bacterial and fungal composition. BCA application affected fungal composition, resulting in a high number of OTUs (as a measure of fungal diversity), which varied significantly among different fungal classes.

In general, the type of BCA applied and its introduction as a single or combined treatment affected bacterial and fungal relative abundance and diversity. The synergetic effect of BCAs as a double or triple treatment increased the microbial diversity; however, only those applications containing Boni were effective to decrease the relative abundance of the *Leotiomycetes*, containing *B. cinerea*. Therefore, the decision on how to apply BCAs should be made with a clear aim of mitigating grey mould rather than enhancing the richness of microbiota. Generation of thousands of reads using a high-throughput sequencing platform does not only indicate phenotypic changes in BCA-treated strawberry fruit, but also helps to evaluate the microbial composition of the fruit microbiome at the molecular level, for better protection during production and storage as well as further consumption.

## ACKNOWLEDGEMENTS

This project was made possible due to financial support of Andre Freire Cruz by the Alexander von Humboldt-Foundation - AvH (Germany). Field experiments in 2011 and 2012 were financially supported by the Federal Ministry of Food, Agriculture and Consumer Protection —BMELV (Germany). Thanks to Erika Krüger and Walter Wohanka for support during field experiments and to AbiTEP GmbH, Koppert Biological Systems, and Intrachem Bio Deutchland GmbH & C. KG for supplying the BCA products. Colleagues of Geisenheim University are acknowledged for their assistance during the experiments.

## ORCID

*Andre Freire Cruz* http://orcid.org/0000-0001-9349-2742
*Geleta Dugassa Barka* http://orcid.org/0000-0002-1521-9705
*Annette Reineke* http://orcid.org/0000-0001-8856-5505

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Cruz AF, Barka GD, Sylla J, Reineke A. Biocontrol of strawberry fruit infected by *Botrytis cinerea*: Effects on the microbial communities on fruit assessed by next-generation sequencing. *J Phytopathol*. 2018;00:1–9. https://doi.org/10.1111/jph.12700