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Searching, identifying and characterizing antagonistic isolates to control *Neofabraea* representatives causing bull's-eye rot in apples**

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Abstract. Apple bull's eye rot (BER), caused by Neofabraea representatives, is a major challenge for apple production. This study aimed to identify microbial antagonists capable of suppressing Neofabraea by leveraging soil microorganisms from six distinct apple orchard land management systems. Key candidates, Bacillus velezensis (B134/22, B233/22, B267/22) and Trichoderma koningiopsis (G779/22) inhibited 65% of Neofabraea isolates, indicating their strong potential for biocontrol. T. koningiopsis produced chitinase and β-glucanase, while B. velezensis relied on glycogen degradation, suggesting diverse biocontrol strategies. Antibiotic resistance profiles showed minimal resistance genes, reducing environmental risks. Sensitivity to fungicides was assessed, with T. koningiopsis sensitive to Siarkol and B. velezensis to Zato and Luna fungicides, supporting their compatibility with orchard practices. Tested isolates showed susceptibility to some chemical substances used in apple orchard practice (e.g. antibiotics, copper(II) sulfate, fusidic acid, or promethazine). These findings highlight the importance of integrating biocontrol agents with agrochemical use, ensuring sustainability in apple production while minimizing environmental impact.

K eywords: biocontrol, antagonist, bull's-eye rot, *Neofabraea, Bacillus velezensis, Trichoderma koningiopsis*, apple, soil, whole genome sequencing

1. INTRODUCTION

With over 77 million t produced in 2023/2024, apples rank as the fourth most significant fruit produced and consumed globally (Musacchi and Serra, 2018). Apples are a natural product with healing properties that are successfully used in the prevention of many diseases (Łysiak and Szot, 2023). Given their significance in both industry and agriculture, it is crucial to ensure that their quality remains stable throughout storage (Ahmadi-Afzadi et al., 2013; Sottocornola et al., 2022; Lipa et al., 2019). One of the challenges faced by fruit growers is the prevalence of postharvest diseases. Among these, Bull's Eye Rot (BER), caused by fungi of the genus Neofabraea (syn. Pezicula, Phlyctema, Gloeosporium), is particularly significant (Cao et al., 2013; Lin et al., 2018; Oszust et al., 2023a). One way to combat fungal pathogens that cause BER is to use synthetic fungicides. Some of these compounds are currently approved for use, but the main threat is that they exhibit broad-spectrum activity against various microorganisms (Brauer et al., 2019; Zia et al., 2022). Examples of synthetic fungicides used against BER include active substances such as captan or thiophanate-methyl (Aguilar et al., 2018; Wood and Fisher, 2017). The European Union plans to discontinue the production and use of these substances (Silva et al., 2022). Fungicide chemicals have

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a long withdrawal period and may contribute to the decrease of microbial biodiversity in the environment (Oleszek et al., 2019). Oszust et al. (2023a), in their recent review, emphasize approaches promoted by the European Commission - such as those outlined in the Green Deal and the Union Biodiversity Strategy - that aim to ensure food safety and foster more sustainable agricultural development. These approaches encourage sustainable agricultural practices that reduce environmental impact, mitigate climate change, and enhance food safety. Additionally, the European Commission aims to reduce the use of chemical plant protection products and the associated risks by 50% by 2030. Thus, seeking more environmentally sustainable solutions is essential to align with the objectives of the European Green Deal and support biodiversity protection (Bryk and Rutkowski, 2012; Oszust et al., 2023a).

The use of biopreparations containing microorganisms is considered a beneficial solution in this context (Oszust et al., 2023a, b). Biopreparations might serve as an alternative to chemical plant protection products, offering greater safety for human health, animals and the environment. Additionally, microorganisms are usually highly selective for specific pathogens (Grishechkina et al., 2019). Available biopreparations contain generally biologically active ingredients that are based mostly on non-GMO microorganisms belonging to the groups of bacteria, yeasts, and filamentous fungi or their metabolites and exhibit antagonistic effects towards pathogens (Grzegorczyk et al., 2015; Pylak et al., 2019). The greatest interest in the biocontrol of pathogens is aroused by bacteria representatives of the genus Bacillus and fungi of the genus Trichoderma (Oszust et al., 2023a). Trichoderma fungi are common in all soils and inhabit plant roots, strengthening their defense against pathogens. They exhibit antagonistic activity against many microorganisms and are known for their rapid growth. The action of Trichoderma against pathogens is based on competition for nutrients and living space, as well as on the mechanism of mycoparasitism (Oszust et al., 2021; Patel et al., 2019). Bacteria of the genus Bacillus, belonging to the group of Gram-positive bacteria, are also often used in biocontrol due to their ability to form endospores and the wide spectrum of action of the compounds they produce, for example, antibiotics or various antimicrobial peptides (Fira et al., 2018; Kumbar et al., 2019). A new approach that gives positive results is the construction of two-component biopreparations, which include microorganisms from two different groups (Poveda and Eugui, 2022). Moreover, studies suggest that locally sourced microorganisms exhibit greater efficacy against indigenous pathogens compared to those sourced from other regions (Oszust et al., 2021; Pylak et al., 2020). This finding may prove advantageous, particularly in the development of biopreparations and in enhancing their effectiveness (Derikvand et al., 2023; Zydlik et al., 2021).

This research aimed to isolate, identify, and characterize antagonistic isolates of bacteria from the genus Bacillus and fungi from the genus Trichoderma for their potential to combat Neofabraea spp. Additionally, the study sought to select isolates that could serve as suitable candidates for a biopreparation consortium against Bull's Eye Rot (BER). Based on these objectives, we formulated the following hypotheses: (i) soils beneath apple trees will be a suitable source for isolating Bacillus spp. and Trichoderma spp. isolates with antagonistic properties against Neofabraea spp., enabling the selection of candidates for a future biopreparation consortium to combat BER, and (ii) genetic identification, characterization of antagonistic properties, functional traits, chemical sensitivity, and fungicide resistance will provide a comprehensive understanding of the selected isolates.

2. MATERIALS AND METHODS

2.1. Obtaining microbial isolates from soils beneath apple trees

Soil samples were collected from apple trees growing in six different land management practices: hedgerows (belts) separating cultivated fields (B), forest areas (F), gardens with ornamental plants and fruit trees (G), courtyards/gardens where farm animals are kept (GA), orchards where the use of agrochemical treatments has been discontinued, uncultivated orchards (OU), and orchards cultivated in an integrated system (OC) according to the method described by Zawadzka *et al.* (2024).

To obtain pure cultures of microorganisms, a series of dilutions of a soil suspension from apple orchard soils was plated on enriched agar media. Bacterial isolates were obtained from a selective agar medium using tryptone soy agar (TSA), which was intended to isolate microorganisms mostly of the genus *Bacillus*. Fungal isolates were sought on *Trichoderma* selective medium (TSM) to target these fungi specifically.

Both agar media were enriched with a mixture of necromass and post-culture fluid derived from the mycelial biomass of eight *Neofabraea* spp. These isolates were obtained from apples displaying Bull's Eye Rot (BER) symptoms. *Neofabraea* spp. cultures were prepared by cultivating the isolates in a potato dextrose broth (PDB), a liquid medium at 19°C for 7-9 days. The necromass and post-culture fluid were then added to the selective media at a 5% concentration each, allowing for the growth of microorganisms in the presence of metabolites produced by *Neofabraea* spp. and cellular components. These enriched selective media were designed to pre-select isolates with potential antagonistic properties against *Neofabraea* spp.

The culture in the flasks was poured into Falcon tubes and centrifuged for 3 min at 3200 rcf. Then the supernatant was filtered through a filter paper and filtered through a microbiological filter (pore size 0.22μ m), and the pellet

was transferred to a beaker and homogenized with a goblet blender. Then the homogenate was autoclaved for 20 min at 136°C. Sterile necromass homogenate and post-culture fluid filtrate were added to sterile media prepared in flasks and poured onto sterile Petri dishes. Bacterial isolates were cultured on plate count agar (PCA medium) and fungi isolates on potato dextrose agar (PDA medium). Pure cultures of bacteria were selected by preliminarily identified based on the appearance of the colonies as belonging to the genus Bacillus, as opposed to Pseudomonas and also based on Gram staining - differences in staining between Gram-positive bacteria, which include Bacillus spp., and Gram-negative bacteria. The fungal isolates were selected by preliminarily, macromorphologically identified as belonging to the genus Trichoderma. The cultures were incubated for 3-7 days at 24°C.

2.2. Stepwise selection of effective bacterial and fungal antagonists of *Neofabraea* spp.

The antagonistic properties of the obtained isolates were evaluated to select candidates for a biopreparation consortium targeting *Neofabraea* phytopathogens. Plate test analyses were performed and the level of antagonism, followed by the ability of microbial isolates to limit the growth of *Neofabraea* spp. was assessed. The first stage of these tests included 43 bacterial isolates and 31 fungal isolates against seven randomly selected *Neofabraea* spp.

The experiment used plates with a PDA to test selected bacteria's antagonistic properties and a PDA with antibiotics (streptomycin and chloramphenicol, each added at a concentration of 100 µL per mL of medium) for testing fungal isolates for their antagonism (in three replications). Then, the suspension of each isolate of Neofabraea spp. was standardized into 70% transmittance using a turbidimeter (BiologTM). Then 100 µl of a standarised suspension of each Neofabraea spp. were sown on the plate with adequate medium and incubated for 48 h at 18°C. Neofabraea spp. grows best at low temperatures and slower than the tested bacteria or fungi. To ensure reliable results, plates with Neofabraea spp. were pre-incubated before introducing the antagonistic organisms. Next, antagonistic microorganisms were added to the plate. In the case of bacterial isolates, the procedure was as follows: Three holes were cut in the substrate in each plate using sterile 1 ml tips, then 15 µl of bacterial suspension (70% T) was added. For fungi: fungal spores were added to the medium with the addition of antibiotics, also in triplicate, by puncturing with an inoculation loop. The plates were then incubated at 24°C for 48 h (bacteria) and 96 h (fungi) and afterward the zones of growth inhibition were measured.

Following this, six isolates were selected from the bacterial group and six isolates of fungi for which the highest ability to inhibit the growth of *Neofabraea* spp. was noted. In the next stage, the isolates of the selected antagonists were tested against all obtained isolates of *Neofabraea* spp., *i.e.* 150 isolates (a representative set of apple orchards in Poland). The apples belonged to 46 varieties from 57 localities. Finally, three bacterial isolates (B134/22, B233/22, B267/22) and one fungal isolate (G779/22) were selected as the candidates for biopreparation against *Neofabraea* spp.

2.3. Whole Genome Sequencing by Illumina[®] – genetic identification and functional analyses of isolates with the greatest antagonistic properties

Whole genome sequencing (WGS) analysis was performed using Illumina® MiSeq v3 (2x300) technology of four selected, antagonist isolates. The genomic DNA of microorganisms was extracted using the EURx GeneMATRIX Bacterial and Yeast Genomic DNA Purification Kit and EURx GeneMATRIX Plant & Fungi DNA Purification Kit (EURx®, Gdansk, Poland). WGS libraries were prepared with Illumina DNA Prep kit. Raw sequencing bcl data was basecalled with RTA (Illumina) and demultiplexed with bcl2fastq (Illumina), then quality assessment and quality trimming was performed on obtained fastq files with fastQC (Cock et al., 2010; Andrews, 2010) and Cutadapt (Martin, 2011). After that, de novo sequences assembly was performed with SPAdes (Prjibelski et al., 2014; 2020) Genome Assembler and fasta (Pearson and Lipman, 1988) files containing contigs and scaffolds were obtained. QUAST (Gurevich et al., 2013; Mikheenko, 2018) Icarus (Mikheenko et al., 2016), and BUSCO (Simão et al., 2015) analysis and benchmark were performed to assess the quality of assemblies. Bacterial genome annotations were performed with prokka (Seemann, 2014) tool with the addition of resistance gene identifier (RGI) (Alcock et al., 2023) to detect resistance mechanism within bacterial genome, while fungal annotations were performed with funannotate (Love et al., 2018) wrappertool. Within the fungal annotation pipeline, tRNAscan-SE (Lowe and Eddy, 1997) was used to detect tRNA genes, Phobius (Käll et al., 2004) and SignalP 6.0 (Teufel et al., 2022) to predict and identify transmembrane topology and signal peptide, while eggNOG 6 (Hernández-Plaza et al., 2023), InterProScan 5 (Blum et al., 2021; Jones et al., 2014) and antiSMASH 7.0 (Blin et al., 2023) were used to functional annotation and secondary metabolism genes finding. FastANI (Jain et al., 2018) and BLAST (Altschul et al., 1990; Camacho et al., 2009) tools were used against NCBI RefSeq (O'Leary et al., 2016) database to identify studied contigs. KofamKOALA CLI (Aramaki et al., 2020) tool was used to allocate predicted genes of both bacterial and fungal isolates to metabolic pathways and asses their completeness. Whole genome sequencing analysis enabled functional analysis of the genome to determine the biological potential and indicate genes useful in the biocontrol of candidates for the consortium of biopreparations.

2.4. Chemical sensitivity

Experiments were performed to assess the chemical sensitivity of candidates for the biopreparation consortium, namely selected active antagonists from the genus Bacillus and Trichoderma representatives. Phenotypic analyses based on the BiologTM system were used: GENIII plates dedicated to bacterial cultures and PM21D plates suitable for fungal cultures (Biolog[™], Hayward, CA, USA). An appropriate amount of fungal spores or bacterial cells according to the manufacturer's protocol was mixed with the inoculum fluid to obtain transmittance of 81%T for bacteria and 62%T for fungi, respectively. The plates containing lyophilized substrates were inoculated with a 100 µl suspension of microorganisms. The plates were incubated at 24°C and absorbance measurements were taken at 750 nm at 24 h intervals for 10 consecutive days. The wavelength of 750 nm, was used to measure optical density, which correlates with changes in microbial biomass production (Oszust et al., 2023b).

Chemical sensitivity tests were performed on the plates, including cations, organic compounds, fungicides, antibiotics/bacteriocides, cell membrane-toxic substances, salinity, and pH. The GENIII plate included 23 chemical compounds, and the PM21D plate included 24 chemical compounds, grouped according to Panek *et al.* (2016) and Pylak *et al.* (2020). The analyses were carried out under the manufacturer's protocols (Chojniak *et al.*, 2015; Panek *et al.*, 2016).

2.5. Fungicide sensitivity

In this experiment, a range of ten fungicides, namely Siarkol Extra 80WP, Bellis 38 WG, Zato 50 WG, Captan 80WG, Unix 75 WP, Miedzian 50 WP, Delan 700 WG, Geoxe 50 WP, Switch 62,5 WP, Luna Experience 400 SC were prepared in solution at specific concentrations to be applied as in agricultural doses. The final concentrations of the fungicide solutions were as follows: Bellis at 0.16%, Delan at 0.25%, Zato at 0.04%, Captan at 0.4%, Unix at 0.35%, Siarkol at 1.25%, Miedzian at 0.3%, Luna at 0.15%, Geoxe at 0.15%, and Switch at 0.125%.

The fungicide sensitivity test was performed using a well test. Bacterial and fungal isolates were sown on an agar medium. Transmittance (%T) was determined at 70% for all isolates tested. Then, 100 μ l of the material was pipetted onto the plates. Holes were cut out in the medium using a sterile pipette tip and 50 μ l of fungicide at the appropriate concentration was added to the center. The plates were incubated at 30 and 24°C for bacterial and fungal isolates,

respectively. The growth inhibition of *Bacillus velezensis* and *Trichoderma koningiopsis* was measured after 7 days of incubation.

3. RESULTS

3.1. Obtained bacterial and fungal isolates

A total of 296 bacterial isolates and 119 fungal isolates were collected. Isolates of bacteria and fungi obtained from the soil under apple trees are presented in Fig. 1. The graph presents the amounts of *Bacillus* spp. and *Trichoderma* spp. isolates obtained from the soil under apple trees depending on the land management method. Figure 1a shows the number of obtained bacterial isolates, while Fig. 1b shows the number of fungal isolates obtained.



Fig. 1. Number of obtained isolates of bacteria (a) and fungi (b) across different management practices: belts (hedges) separating cultivated fields (B), forest areas (F), gardens with ornamental plants and fruit trees (G), courtyards/gardens where farm animals are kept (GA), orchards where the use of agrochemical treatments has been discontinued, uncultivated orchards (OU), and orchards cultivated in an integrated system (OC).

3.2. Antagonism against Neofabraea representatives

The results of the antagonist tests are presented in Figs 2 and 3. The low degree of growth inhibition of the tested collection of 150 fungi of the genus *Neofabraea* was classified in the range of relatively low (+) 8-12 mm, good (++) 13-15 mm, and very good (+++) >16 mm. Bacteria isolate B134/22 inhibited the growth of 12 *Neofabraea* isolates to a low degree, 45 to a good degree, and 35 to a very good degree. B233/22 inhibited the growth of 12 *Neofabraea*

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isolates to a low degree, 36 to a good degree, and 51 to a very good degree. B267/22 inhibited the growth of 30 *Neofabraea* isolates to a low degree, 33 to a good degree, and 33 to a very good degree.

Fungal isolate G633/22 inhibited the growth of 5 *Neofabraea* isolates to a low degree, 14 to a good degree, and 80 to a very good degree. G756/22 fungal isolate inhibited the growth of 4 isolates to a low degree, 9 to a good degree, and 81 to a very good degree. G779/22 inhibited the growth of 12 *Neofabraea* spp. isolates to a low degree, 11 to a good degree, and 76 to a very good degree.

Finally, three isolates of bacteria (B134/22, B233/22, B267/22) and one isolate of fungi (G779/22), were chosen for the biopreparation consortium as candidates based on the high degree of *Neofabraea* growth inhibition. A total of 65% of the 150 tested *Neofabraea* isolates were inhibited by the antagonistic isolates. Figure 4 presents pictures that confirm the antagonism followed within growth inhibition zones of selected isolates for biopreparation against five arbitrarily selected different *Neofabraea* spp. isolates. The selected isolates underwent further analyses.

3.3. Genetic identification and functional analysis

Table 1 presents the genetic identification of isolates selected from the antagonism tests, based on Whole Genome Sequencing (WGS), along with details on their origins and locations. WGS analysis revealed that all bacterial isolates under consideration for the biopreparation belong to the species *Bacillus velezensis* (B134/22, B233/22, B267/22). In contrast, the fungal isolate G779/22 was identified as the species *Trichoderma koningiopsis*. Identification was through comparison with the GenBank NCBI sequence database. The tested bacterial isolates did not show any differences in the properties revealed by functional analyses following WGS.

Using the KofamKOALA – KEGG Orthology Search tool (Table 2), the completeness of the genome of the tested *Bacillus velezensis* isolates and *Trichoderma koningiopsis* isolate was included in sulfur assimilation, glycogen or carbon degradation, glycolysis, amino acid biosynthesis, and nitrogen metabolism. The presence of genes encoding the production of chitinases and β -glucosidase was found for *Trichoderma koningiopsis*, but not *Bacillus velezensis*. However, these bacteria contained genes encoding starch and glycogen degradation.

Additionally, using the Resistance Gene Identifier (RGI) (Fig. 5) tool, only a few potential antibiotic and disinfectants resistance genes were identified in the genomes of *Bacillus velezensis* isolates due to the presence of the following genes: qacG (quaternary ammonium compound resistance gene), bcl (β -lactamase gene), FosBx1 (fos-fomycin resistance gene), vanT (vancomycin resistance gene), vanG cluster (vancomycin resistance gene), qacJ (quaternary ammonium compound resistance gene), vanY (D,D-carboxypeptidase gene), vanB cluster (vancomycin resistance gene cluster).

3.4. Antagonistic isolates' chemical sensitivity – evaluating resistance

The sensitivity of the tested microorganisms to several different chemical compounds was evaluated and the results are presented in Tables 3 and 4.

Bacillus velezensis showed different levels of sensitivity to the tested chemical compounds. All three tested isolates showed sensitivity to antibiotics: troleandomycin, rifamycin SV, minocycline, lincamycin, niaproof 4, vancomycin, nalidixic acid, and potassium tellurite. Regarding aztreonam, sensitivity was shown only in the first two days of incubation. Guanidine HCl, lithium chloride, sodium bromide, D-serine, tetrazolium violet, fusidic acid, tetrazolium blue, and pH 5 and pH 6 caused a decrease in adsorption of bacterial isolates between 4 and 6 days of incubation, which means that longer exposure to a given chemical compound/culture conditions caused inhibition of bacterial biomass growth. On the other hand, 1% sodium chloride, 4% sodium chloride, 8% sodium chloride, 1% sodium lactate, and sodium butanoate did not cause growth inhibition in the tested isolates, which had an effect on increasing turbidity (T) and thus increasing biomass.

The effect of compounds influencing the chemical sensitivity of the tested Trichoderma koningiopsis isolate can be divided into two groups: substrates that caused a slight, unnoticeable increase in absorbance, and consequently an increase in biomass, and those in which the tested isolate was able to increase biomass production only after 72 h of incubation, i.e. its sensitivity to compounds contained on the plate increased after a certain time of exposure. Chemical compounds such as promethazine, dodecyltrimethyl ammonium bromide, cetylpyridinium chloride, domiphen bromide, sodium dichromate, magnesium chloride, copper(II) sulfate, trifluoperazine, thiourea, zinc chloride caused visible sensitivity of the tested Trichoderma koningiopsis isolate, affecting the inability to increase cell biomass in the presence of these compounds. Guanidine hydrochloride, 2,2'-dipyridyl, nystatin, protamine sulfate, L-aspartic acid b-hydroxamate, 1-hydroxypyridine-2-thione, EDTA, Compound 48/80, manganese (II) chloride, neomycin, D-cycloserine, sodium selenite, nickel chloride, diamide are compounds to which the tested fungal isolate showed chemical sensitivity only in the initial days of incubation.

It can be observed that compounds from the cation groups mostly influenced the limitation of the biomass growth of the tested fungi, while substrates belonging to groups such as chelators or antibiotics sensitized the tested



Fig. 4. Pictures on antagonistic activity in growth inhibition zones of biocontrol biopreparation candidates – *Bacillus velezensis* and *Trichoderma koningiopsis* isolates against selected *Neofabraea* spp.

Trichoderma koningiopsis isolate only for a certain period, which means that it was able to adapt to the action of unfavorable compounds.

3.5. Antagonists' response to fungicides

The study's findings highlight the varying efficacy of different fungicides in inhibiting fungal and bacterial growth. Figure 6a presents the inhibition of *Trichoderma koningiopsis* G779/22. In this case, Siarkol stands out as the most effective fungicide, achieving an inhibition zone of approximately 28 mm, significantly surpassing all other treatments. This strong inhibitory effect is distinct. Unix also demonstrated considerable effectiveness with an inhibition zone of about 22 mm, followed by Bellis, which showed moderate activity at around 17 mm. Meanwhile, Zato and Captan exhibited similar inhibition levels, ranging from 12 to 13 mm. In contrast, Delan, Miedzian, Geoxe, Luna, Switch, and the control treatment displayed negligible inhibition, all grouped under the same category.



Fig. 5. Antibiotic resistance genes of *Neofabraea* biocontrol biopreparation candidates – *Bacillus* velezensis isolates.

Figure 6b focuses on the inhibition of three isolates of Bacillus velezensis (B134/22, B233/22, and B267/22), revealing a different pattern of effectiveness among the fungicides. For isolate B134/22, Zato and Luna emerged as the leading fungicides, achieving inhibition zones of approximately 17 to 18 mm. Other treatments, including Bellis, Delan, Miedzian, Geoxe, Captan, Unix, Siarkol, and the Control, showed little to no impact. Isolate B233/22 revealed a notable result with Siarkol displaying the highest inhibition at around 12 mm. Fungicides, such as Zato, Luna, and Switch demonstrated moderate inhibition, ranging from 5 to 7 mm, while the remaining treatments, including Bellis, Delan, Miedzian, Geoxe, Captan, Unix, and control, showed no significant effect. In the case of isolate B267/22, Captan emerged as the most effective fungicide, achieving an inhibition zone of approximately 16 mm. A group of fungicides including Zato, Luna, and Switch exhibited moderate inhibition (7-8 mm), while the rest of the treatments - Bellis, Delan, Miedzian, Geoxe, Unix, Siarkol, and control showed minimal or no inhibitory effect respectively.

Table 1. Datasheet on Neofabraea biocontrol biopreparation candidate microbial isolates

Isolate	Genetic identification	Sequence in GenBank No.	Apple variety	Land management practice	Localization (latitude N/ longitude E)
B134/22	Bacillus velezensis	PQ620373	Antonówka	Garden	51°03'55.0"N 22°56'11.1"E (51.065272, 22.936407)
B233/22	Bacillus velezensis	PQ620374	nd	Garden	51°00'03.2"N 22°15'20.7"E (51.000889, 22.255750)
B267/22	Bacillus velezensis	PQ620375	Sztetyna	Garden and Animals	51°05'21.2"N 22°43'53.7"E (51.089213, 22.731569)
G779/22	Trichoderma koningiopsis	PQ620377	Kronselka	Bounds	51°22'02.1"N 22°16'19.2"E (51.367237, 22.271997)

Function	B134/22	B233/22	B267/22	Function	G779/22
Retinal biosynthesis	0.25	0.25	0.25	4-Hydroxybutyrate/3- hydroxypropionate	0.10
Biofilm PGA Synthesis protein	0.25	0.25	0.25	Competence-related core components	0.14
4-Hydroxybutyrate/3-hydroxypropionate	0.30	0.30	0.30	Mixed acid: Formate to CO_2 & H_2	0.17
Mixed acid: PEP to Succinate via OAA, malate & fumarate	0.40	0.40	0.40	Polyhydroxybutyrate synthesis	0.17
Serine pathway/formaldehyde assimilation	0.40	0.40	0.40	Staphyloaxanthin biosynthesis	0.17
Twin Arginine Targeting	0.50	0.50	0.50	Serine pathway/formaldehyde assimilation	0.20
Bidirectional polyphosphate	0.50	0.50	0.50	Arsenic reduction	0.25
TCA Cycle	0.62	0.62	0.62	Starch/glycogen synthesis	0.33
Cytochrome c oxidase	0.75	0.75	0.75	End-product myxoxanthophylls	0.33
Entner-Doudoroff Pathway	0.75	0.75	0.75	NADH-quinone oxidoreductase	0.35
Anaplerotic genes	0.75	0.75	0.75	F-type ATPase	0.38
Arsenic reduction	0.75	0.75	0.75	Mixed acid: PEP to Succinate via OAA, malate & fumarate	0.4
Competence-related related components	0.80	0.80	0.80	Thiamin biosynthesis	0.45
Sec-SRP	0.81	0.82	0.83	TCA Cycle	0.50
Valine	0.83	0.83	0.83	Riboflavin biosynthesis	0.50
Isoleucine	0.83	0.83	0.83	Entner-Doudoroff Pathway	0.50
Flagellum	0.87	0.87	0.87	Bidirectional polyphosphate	0.50
Chemotaxis	0.88	0.88	0.88	Ubiquinol-cytochrome c reductase	0.66
Glycolysis	0.89	0.89	0.89	Valine	0.66
Competence-related core components	0.98	0.98	0.98	Isoleucine	0.66
MEP-DOXP pathway	0.99	0.99	0.99	Anaplerotic genes	0.75
F-type ATPase	1.00	1.00	1.00	Mevalonate pathway	0.80
Cytochrome aa3-600 menaquinol oxidase	1.00	1.00	1.00	Glycolysis	0.89
Cytochrome bd complex	1.00	1.00	1.00	Gluconeogenesis	0.89
D-galacturonate isomerase	1.00	1.00	1.00	Beta-N-acetylhexosaminidase	1.00
Alpha-amylase	1.00	1.00	1.00	Alpha-amylase	1.00
Nitrite oxidation	1.00	1.00	1.00	Beta-glucosidase	1.00
Dissimilatory nitrate reduction	1.00	1.00	1.00	Dissimilatory sulfate <> APS	1.00
Dissimilatory sulfate <> APS	1.00	1.00	1.00	Sulfide oxidation	1.00
Sulfur assimilation	1.00	1.00	1.00	Sulfur assimilation	1.00
Thiamin biosynthesis	1.00	1.00	1.00	Alcohol oxidase	1.00
Riboflavin biosynthesis	1.00	1.00	1.00	Mixed acid: Acetate	1.00
Transporter: phosphate	1.00	1.00	1.00	Mixed acid: Ethanol, Acetate to Acetylaldehyde	1.00
Mixed acid: Lactate	1.00	1.00	1.00	Glyoxylate shunt	1.00
Mixed acid: Acetate	1.00	1.00	1.00	Histidine	1.00

Table 2. Genome completeness of Neofabraea biocontrol biopreparation candidates – Bacillus velezensis and Trichoderma koningiopsis isolates following KofamKOALA - KEGG Orthology Search tool

Value of 1 indicates a complete genome for the given function, while a value < 1 indicates an incomplete genome.

Table 2. Continuation

Function	B134/22	B233/22	B267/22	Function	G779/22
Mixed acid: Ethanol, Acetate to Acetylaldehyde	1.00	1.00	1.00	Arginine	1.00
Cobalt transporter CorA	1.00	1.00	1.00	Lysine	1.00
Copper transporter CopA	1.00	1.00	1.00	Serine	1.00
Fe-Mn transporter MntH	1.00	1.00	1.00	Threonine	1.00
Histidine	1.00	1.00	1.00	Asparagine	1.00
Arginine	1.00	1.00	1.00	Glutamine	1.00
Lysine	1.00	1.00	1.00	Cysteine	1.00
Serine	1.00	1.00	1.00	Glycine	1.00
Threonine	1.00	1.00	1.00	Proline	1.00
Asparagine	1.00	1.00	1.00	Methionine	1.00
Glutamine	1.00	1.00	1.00	Phenylalanine	1.00
Cysteine	1.00	1.00	1.00	Tyrosine	1.00
Glycine	1.00	1.00	1.00	Aspartate	1.00
Proline	1.00	1.00	1.00	Glutamate	1.00
Alanine	1.00	1.00	1.00	Starch/glycogen degradation	1.00
Methionine	1.00	1.00	1.00	Glucoamylase	1.00
Phenylalanine	1.00	1.00	1.00	Cellulase	1.00
Leucine	1.00	1.00	1.00	Chitinase	1.00
Tryptophan	1.00	1.00	1.00	Cobalt transporter CorA	1.00
Aspartate	1.00	1.00	1.00	Copper transporter CopA	1.00
Glutamate	1.00	1.00	1.00	Leucine	1.00
Starch/glycogen degradation	1.00	1.00	1.00	Tryptophan	1.00

Overall, the results indicate that Siarkol is the most potent fungicide against *Trichoderma koningiopsis*. Meanwhile, Zato and Luna demonstrate consistent effectiveness across various isolates of *Bacillus velezensis*. Some fungicides, such as Bellis, Delan, Miedzian, and Geoxe show minimal to no significant impact on either microorganism, suggesting limited efficacy in these contexts.

4. DISCUSSION

4.1. Phenomena underlying robust search for *Neofabraea* microbial antagonists in soil

Based on our hypothesis, a few microbial isolates collected from the soil beneath apple trees exhibit antagonistic properties that could be utilized for biocontrol of *Neofabraea* isolates responsible for causing apple Bull's Eye Rot (BER). We selected soil from beneath apple trees for our search, adopting the approach commonly used in the search for antagonists against other fungal phytopathogens (Oszust *et al.*, 2021; Pylak *et al.*, 2020). What unites these studies is the underlying phenomenon: the natural interactions between soil microorganisms, which can lead to the emergence of antagonistic properties. In the soil environment, microorganisms are in constant interaction, competing for resources and niches, which may trigger the development of mechanisms that facilitate their coexistence - such as the production of antimicrobial compounds or competitive exclusion (Tyc et al., 2014; Whipps, 2001; Zhang et al., 2021). This continuous microbial interaction increases the likelihood of isolating isolates that possess the desired biocontrol activity. Indeed, Neofabraea is primarily associated with the presence of fruits, but it is not limited to them. This pathogen also inhabits the bark of apple trees (Aguilar et al., 2019; Gariépy et al., 2003), and based on our unpublished observations, it is also present in the soil beneath the trees. The widespread distribution of Neofabraea across different parts of the tree presents an opportunity to search for antagonistic microorganisms not only on the fruit but also in the soil, and thus, this approach was followed.

In light of previous studies evaluating the impact of different land management practices on soil bacterial and fungal communities under apple trees, it is clear that soil microbiomes can be significantly influenced by management (Zawadzka *et al.*, 2024). A study examining soil



Table 3. Chemical sensitivity of *Neofabraea* bacterial biocontrol biopreparation candidates – *Bacillus velezensis* isolates on GENIII

 BiologTM plate within time of incubation

The color scale ranges from blue to green, where darker blue indicates higher sensitivity, while green represents a lack of sensitivity (both in relation to the positive control).

					Time (h	n)			
Chemical compound	0	24	48	72	96	120	144	168	192
		Anion	IS						
Sodium dichromate									
Sodium selenite									
		Antibio	tcs						
Neomycin									
D-cycloserine									
		Cation	ıs						
Dodecyltrimethyl ammonium bromide									
Manganese (II) chloride									
Magnesium chloride									
Copper (II) sulfate									
Nickel chloride									
Zinc chloride									
		Chelato	ors						
2,2'-dipyridyl									
1-hydroxypyridine-2-thione									
EDTA									
	Cy	yclic com	pounds						
Promethazine									
Compound 48/80									
	Membra	ne functio	n compo	unds					
Guanidine hydrochloride									
Nystatin									
Protamine sulfate									
Cetylpyridinium chloride									
Domiphen bromide									
	Nit	rogen con	npounds						
Diamide									
Thiourea									
		Hydroxan	nates						
L-aspartic acid β-hydroxamate									
	Heter	ocyclic co	ompound	s					
Trifluoperazine									

Table 4. Chemical sensitivity of *Neofabraea* biocontrol biopreparation fungal candidate – *Trichoderma koningiopsis* isolate on PM21 BiologTM plate within time of incubation

The color scale ranges from red through orange and yellow to green, where green indicates lack of sensitivity (in relation to the positive control).

beneath apple trees growing in six different management practices demonstrated that these practices indeed impact the soil microbiome properties. These variations in microbial communities were important to consider if they positively affect the presence and diversity of antagonistic microorganisms capable of biocontrol of *Neofabraea*.

A key phenomenon driving the search for *Neofabraea* antagonists is thus the diversity among microbial isolates of the same species, as well as the intra- and interspecific diversity among pathogens themselves (Pertile *et al.*, 2018).

This diversity stems from various factors, including genetic variation, environmental conditions, and geographical origin. Genetic differences can lead to variations in traits like antibiotic resistance and metabolic capabilities, while environmental factors such as temperature, pH, and nutrient availability influence microbial behavior and antagonistic potential (Baquero *et al.*, 2021; Depardieu *et al.*, 2007; Jiménez-Delgadillo *et al.*, 2018; Orr and Nelson, 2018). Additionally, isolates from different locations may adapt to local conditions (Kraemer and Boynton, 2017; Lankau,



Fig. 6. Efficacy of fungicides in inhibiting growth of *Neofabraea* biocontrol biopreparation candidates – *Bacillus velezensis* (a) and *Trichoderma koningiopsis* (b) isolates.

2013; Martignoni and Kolodny, 2024), affecting their ability to combat pathogens like *Neofabraea*. The diversity within *Neofabraea*, as well as the broader diversity among related pathogens, can also influence their pathogenicity and resistance to biocontrol agents. Interactions with specific hosts can shape both microbial and pathogen traits, enhancing the effectiveness of biocontrol strategies. Understanding these factors was crucial for identifying effective antagonists and designing targeted treatments.

In this study, we evaluated the antagonistic potential of various microbial isolates against 150 isolates of *Neofabraea* collected from different orchards, locations, and apple varieties. Our results demonstrated significant variability in the efficacy of the tested antagonists, highlighting the importance of considering both microbial and pathogen diversity when selecting potential biocontrol agents. However, this wide approach is a less common strategy due to its highly time- and labor-intensive nature. Among the 150 *Neofabraea* isolates, a diverse range of responses to the antagonists was observed. Some isolates exhibited strong inhibitory effects, while others showed limited or no antagonistic activity. This suggests that the local environmental conditions and the specific host-pathogen interactions may play a key role in determining the success of biocontrol agents. Our findings are in line with previous studies that highlight the variability of microbial behavior across different ecological niches (Bonaterra *et al.*, 2022; Gómez *et al.*, 2016).

Bacillus velezensis isolates B134/22, B233/22, B267/22, and the fungal isolate G779/22, identified as *Trichoderma koningiopsis*, were selected in our stepwise research due to their significant potential in suppressing 65% of 150 *Neofabraea* isolates collected from Polish apple orchards. These promising results highlight the potential of these candidate isolates for future development into effective biocontrol biopreparations.

Bacillus velezensis is commonly used in biocontrol due to its ability to grow under biotic and abiotic stress conditions, adapt to environmental conditions, and produce spores or compounds that affect plant growth (Alenezi *et al.*, 2021). So far, its ability to combat various fungal pathogens has been demonstrated, including *Fusarium graminearum* or *Botrytis cinerea*, which cause diseases such as *Fusarium* head blight (FHB) and Plant gray mold, respectively (Chen *et al.*, 2018; Jiang *et al.*, 2018). The potential of *Bacillus velezensis* for biocontrol is also visible concerning fungal pathogens causing apple diseases. *Valsa mali* causing apple Valsa canker and *Botryosphaeria dothidea* causing postharvest apple ring rot showed sensitivity to the antagonistic effect of *Bacillus velezensis*, manifested by, among others: growth inhibition or hyphal deformation (Liu *et al.*, 2021; Yuan *et al.*, 2022).

Trichoderma koningiopsis is also used in biocontrol. It has various mechanisms of action, including the ability to grow rapidly, which results in it winning the competition for nutrients due to the intensive multiplication of its cells (Luo *et al.*, 2023). Its antagonistic effect has been confirmed against various pathogens, including *Colletotrichum gloeosporioides* causing the postharvest anthracnose of chili pepper or *Calonectria pseudonaviculata* causing boxwood blight (Kong and Hong, 2017; Ruangwong *et al.*, 2021). It is also used to inhibit the growth of pathogens attacking fruit, *e.g. Fusarium oxysporum* which causes numerous losses in banana cultivation (Luo *et al.*, 2023).

In light of the examples cited, it is worth noting that the combined action of *Bacillus velezensis* and *Trichoderma koningiopsis* has not been used so far to biocontrol fungal pathogens attacking apple trees, especially pathogens of the *Neofabraea* genus, which is an approach proposed for the first time.

4.2. Microbiological characteristics of biocontrol candidates *vs.* apple orchard practices

4.2.1. Linking biocontrol properties to chitinase and β-glucanase

Although differences in antagonistic properties important in the biocontrol of Neofabraea were noted (based on growth inhibition), no genetic differences were found among the tested bacterial isolates (functional analysis of the genomes). The genomes of the Bacillus velezensis isolates did not contain genes for chitinase and β-glucanase production. However, they did harbor genes involved in e.g. the degradation of glycogen, thus obtaining antagonistic properties should result from different characteristics. Antagonistic Bacillus velezensis, particularly those used as biocontrol agents, are often subjected to nutrient-limiting conditions, particularly in soil environments or when competing with plant pathogens (Alenezi et al., 2021; Jiang et al., 2018). On the other hand, what is important, the genome analysis of Trichoderma koningiopsis isolate revealed the presence of genes responsible for the production of chitinases and β-glucosidase, confirming its antagonistic activity against Neofabraea isolates. Our findings followed functional analyses by the WGS indirectly

explain the mechanisms of antagonistic action confirmed *in vitro* antagonistic tests (Corral-Ramos and Roncero, 2015; Derikvand *et al.*, 2023; Palumbo *et al.*, 2005; Wang *et al.*, 2020).

4.2.2. Low risk of antibiotic resistance spread in apple orchard microbial communities

Only a few potential antibiotic-resistance genes were found in the *Bacillus velezensis* genome, driven by several key genes, which situation is crucial for their use in orchard environments. Resistance mechanisms enable *Bacillus velezensis* to withstand a variety of antimicrobial agents (Jian *et al.*, 2021; Berić, 2018; Wash *et al.*, 2022). These were as follows quaternary ammonium compound resistance, β -lactam degradation, fosfomycin inactivation, chloramphenicol neutralization, and vancomycin resistance were found in our investigation (Kanehisa and Goto, 2000).

Although, WGS analysis detected vancomycin resistance genes, *in vitro* sensitivity following PM plates for vancomycin occurred. The noted situation explains that for the tested isolates those genes might not have been expressed or functional. Factors like inducible resistance, gene regulation, or even testing particular conditions could lead to this discrepancy. Thus, the WGS detected the genetic potential for resistance, while culturing tests revealed the actual behavior under tested conditions.

What needs to be highlighted is, that the results showed that these isolates contain fewer resistance genes compared to other isolates, e.g. as presented by Jin et al. (2024) who showed more than ten antibiotic resistance genes in the tested Bacillus sp. isolates. This is particularly significant because even environmental isolates typically harbor a substantial number of resistance genes (Berić, 2018). This feature provides Bacillus velezensis isolates with an advantage for future orchard applications, as their introduction into orchards would be less likely to lead to the spread or transfer of resistance genes within the microbial community (Jian et al., 2021; Zhai et al., 2023). Such studies are crucial before introducing an isolate as part of a biopreparation into the environment, as they help assess the potential risks associated with the spread of antibiotic resistance genes. If biopreparations contain isolates with a high resistance gene content, there is a risk that these genes could transfer to other microorganisms in the environment within the microbial community, potentially leading to the emergence of greater resistance in the environment (Courvalin, 1994; Dai et al., 2012; Zhai et al., 2023). By thoroughly evaluating the resistance profiles of isolates in advance, it is theoretically ensured that the introduction of biopreparations into apple orchards would not unintentionally foster

the spread of resistance, thus promoting the safe and effective use of these isolates in agricultural and environmental applications.

4.2.3. The relevance of sensitivity/resistance properties in enduring agrochemicals used in apple production

The relevance of sensitivity or resistance properties of selected antagonistic isolates in enduring apple production practices should be considered towards agrochemicals commonly applied to the foliage (leaves or stems), whether used in integrative (chemical) or ecological (organic) methods. This is important because these isolates are also intended for foliar application to protect apples against Neofabraea occurrence. Thus, the results on the sensitivity of selected isolates of Bacillus velezensis and Trichoderma koningiopsis to various chemical compounds in fungicides are also relevant in the proper efficiency of biocontrol of Neofabraea in apple orchards. In other words, when sensitivity is observed, special caution should provided to avoid applying these isolates when other substances remain active (i.e. when the withdrawal period of the particular substance has not already passed).

The adaptability of our microbial isolates to different substances suggests that they can survive and remain effective in diverse and potentially stressful orchard environments (Jin *et al.*, 2023; Mohammadi *et al.*, 2023). For example, *Bacillus velezensis* isolates showed resilience to salinity. Similarly, the ability of *Trichoderma koningiopsis* to adapt to certain compounds over time suggests it could withstand fluctuating conditions in the orchard, which is crucial for long-term biocontrol. When used as a biocontrol agent as part of a foliar treatment in apple orchards, it may be exposed to salts from the chemicals applied to the leaves (*e.g.*, potassium salts, sodium chloride, or other salts in the spray solution) (Wani *et al.*, 2022).

On the other hand, tested isolates showed sensitivity to some chemical substances used in apple orchard practice (e.g. antibiotics, copper(II) sulfate, fusidic acid, or promethazine). Streptomycin and oxytetracycline are sometimes used in apple orchards to manage bacterial diseases like fire blight. Although specific antibiotics like rifamycin, minocycline, and vancomycin are not typically used in apple production, sensitivity to antibiotics, in general, can indicate how bacteria might respond to antimicrobial treatments in the orchard environment. Next, copper(II) sulfate is a common fungicide used in apple orchards to control various fungal diseases. The sensitivity of Trichoderma koningiopsis isolate to copper(II) sulfate is relevant because exposure to copper-based fungicides could impact the efficacy of microbial biocontrol agents (Johnson et al., 2023; Kurnik et al., 2012; Mayerhofer et al., 2009).

Similar results of sensitivity were observed for pH. This aspect is important with pH adjusters are applied directly on trees in orchard management, typically with chemical treatments. These are often used to influence the pH of the plant

surface or to optimize the effectiveness of treatments like pesticides, fungicides, or foliar sprays. pH adjusters can be added to spray solutions to ensure that other pesticides or fungicides work effectively. Many agrochemicals are most effective at specific pH levels (Nicholls, 1988; Zhang et al., 2018). Some fungicides are more effective in acidic conditions, while others perform better in alkaline environments. Adjusting the pH of foliar spray agrochemical solutions improves chemical efficacy, enhances absorption, and prevents leaf burn (Spadotto and Hornsby, 2003). Chelated micronutrients like iron, zinc, and manganese are better absorbed when the pH is slightly acidic. Additionally, adjusting pH prevents precipitation of chemicals, ensuring they stay in solution. Citric or acetic acid can lower pH, while sodium hydroxide or potassium hydroxide can raise it, optimizing the treatment's effectiveness (Haynes and Swift, 1985; Madhupriyaa et al., 2024; Naylor et al., 2013; Pampulha and Loureiro-Dias, 1989). Nevertheless, special attention should be paid to pH adjustors may change the efficacy of biocontrol isolates.

In particular, it is also significant to assess the potential for fungicides to reduce the population and activity of these antagonistic isolates. Fungicides can have detrimental effects on the survival and biocontrol potential of microbial agents, which may compromise their ability to protect against pathogens Neofabraea and others. Studies conducted so far have only considered the use of biocontrol agents as a replacement for fungicides, the combined effects of fungicides and biocontrol agents, or a comparison of the effects of both (Lima et al., 2008; Ons et al., 2020; You et al., 2016). This is the first time that the action of fungicides on isolates of antagonistic microorganisms is considered in the laboratory. Therefore, providing detailed information about the sensitivity or resistance of these biocontrol isolates to commonly used fungicides is crucial. The very first step is to test them against fungicides in in vitro tests. We used in our experiments the most commonly applied fungicides (Gupta, 2018; Koller et al., 2005). Briefly, Siarkol emerged as the most potent fungicide against Trichoderma koningiopsis, demonstrating a superior ability to inhibit fungal growth. Meanwhile, Zato and Luna showed consistent effectiveness across tested isolates of Bacillus velezensis, indicating their reliable performance as antibacterial agents. In contrast, Bellis, Delan, Miedzian, and Geoxe exhibited minimal to no significant impact on either Trichoderma koningiopsis or Bacillus velezensis isolates, suggesting that their efficacy is limited in these particular contexts.

This information on the sensitivity/resistance properties of our antagonistic candidates *Bacillus velezensis* isolates B134/22, B233/22, B267/22, and the fungal *Trichoderma koningiopsis* isolate G779/22 against *Neofabraea* on agrochemicals will allow for more informed recommendations on their application strategies, including the timing of fungicide treatments to minimize interference with the activity of the biocontrol agents and maximize their protective effect on apple trees. By considering these characteristics, orchard managers can better integrate biocontrol solutions with chemical or organic management practices. This will help optimize the long-term sustainability of apple production, ensuring that the antagonistic isolates remain effective in controlling *Neofabraea* even in the presence of fungicide treatments.

5. CONCLUSIONS

The study focuses on identifying microbial antagonists against *Neofabraea*, a pathogen responsible for apple Bull's Eye Rot (BER). Soil samples from beneath apple trees growing in six different land management practices were chosen to search for biocontrol agents, leveraging the local and natural interactions of soil microorganisms.

The study evaluated the antagonistic potential of microbial isolates against 150 *Neofabraea* isolates from various orchards and apple varieties. Significant variability in antagonist efficacy highlighted the need to consider both microbial and pathogen diversity in biocontrol selection.

Key candidates identified were *Bacillus velezensis* isolates B134/22, B233/22, B267/22 and a *Trichoderma koningiopsis* isolate G779/22. These isolates suppressed 65% of tested *Neofabraea* representatives, suggesting strong potential for future biocontrol biopreparation development.

Microbial traits linked to biocontrol efficacy, such as chitinase and β -glucanase production, were explored among these isolates. While *T. koningiopsis* G779/22 demonstrated genetic capabilities for these enzymes, *B.velezensis* B134/22, B233/22, and B267/22 relied, among other mechanisms, on glycogen degradation, suggesting other mechanisms contribute to their antagonistic activity.

Importantly, the study examined the antibiotic resistance profiles of the candidates, with only a few resistance genes, which indicates a lower risk of resistance gene spread, making the isolates safer for environmental use.

Sensitivity to agrochemicals was also assessed, given the need for biocontrol agents to endure orchard conditions, including exposure to commonly used fungicides and pH adjusters. The study identified Siarkol as particularly effective against *T. koningiopsis* G779/22, while Zato and Luna were reliable against *B velezensis* B134/22, B233/22, B267/22. In contrast, Bellis, Delan, Miedzian, and Geoxe showed limited efficacy.

Overall, understanding the sensitivity and resistance properties of biocontrol agents is crucial for integrating them with common orchard management practices. This knowledge enables optimized timing and strategies for fungicide applications, ensuring the biocontrol agents remain effective and contribute to sustainable apple production.

The most important conclusions of the study are that *Bacillus velezensis* isolates B134/22, B233/22, B267/22 and *Trichoderma koningiopsis* isolate G779/22 demon-

strated diverse antagonistic mechanisms, combined with favorable antibiotic resistance profiles and sensitivity to agrochemicals, make them promising candidates for developing environmentally safe and sustainable biocontrol solutions against *Neofabraea* spp. in apple orchards.

Conflict of interest. The Authors declare they have no conflict of interest.

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