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Molecular approach for the detection of potential phytopathogens of strawberry plants: PCR assay using functional genes in microbial strains, and environmental samples**

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Abstract. As the microbial quality of fresh fruits and food by-products is relevant, in this study we focus on the development of the detection methods of strawberry phytopathogens: *Colletotrichum* sp., *Phytophthora* sp., *Verticillium* sp., and *Botrytis cinerea*. The primers were designed based on β-tubulin for *Phytophthora* sp., actin for *Verticillium* sp., and glyceraldehyde-3-phosphate dehydrogenase for *Colletotrichum* sp. and *B. cinerea*. The primers were used successively on pure microbial strains, as well as on naturally and artificially contaminated soil and strawberry plant samples. The detection limit for artificially contaminated soil was as follows: *B. cinerea* (100-10 000 spores g^{-1} of soil), *Colletotrichum* sp. (1 000-100 000 spores g^{-1} of soil), and *Verticillium* sp. (100 000 spores g^{-1} of soil), whereas for artificially contaminated strawberry, it was the same for all tested pathogens (100 spores g^{-1} of strawberry). The same primers were used to test bio-preparations, reducing the presence of these potential phytopathogens within different strawberry plant cultivars. The results demonstrated that a developed assay using specific primers designed based on functional genes can be used as a molecular alternative for monitoring and routine investigation of samples contaminated by four important fungal and fungal-like strawberry plant pathogens, including assessing environmental samples such as soil and plants (roots, shoots, fruits).

Keywords: artificially contaminated and environmental samples, potential plant pathogens; strawberry; sustainable agriculture; soil; plant and fruits

1. INTRODUCTION

Before the advent of the molecular era (with Polymerase Chain Reaction, PCR, and Real-Time Polymerase Chain Reaction, qPCR), the identification of phytopathogens was performed based on morphological features (*e.g.* mycelium and spore formation and shape) and also phenotypic characteristics (*e.g.* cultivation media, the decomposition of different chemical species). However, identification based on phenotype characteristics may not be very reliable because these characteristics can change depending on the prevailing environmental conditions (Bragança *et al.*, 2016). Specific PCR protocols required to identify numerous fungal pathogens were proposed, including *Phytophthora fragariae* (Bonants *et al.*, 1997), *Verticillium* sp. (Nazar *et al.*, 1991), *Talaromyces flavus* (Panek and Frąc, 2018), *Neosartorya fischeri* (Pertile *et al.*, 2020; Yaguchi *et al.*, 2012), and *Byssochlamys* sp. (Hosoya *et al.*, 2012; Nakayama *et al.*, 2010).

This study focused on the detection of four economically important fungal and fungal-like pathogens of strawberry plants: *Colletotrichum* sp., *Phytophthora* sp., *Verticillium* sp. and *Botrytis cinerea*. These potential phytopathogens are responsible for significant losses in the harvest following strawberry cultivation, as well as causing a decline in the pre- and post-harvest quality of the fruit. *Botrytis cinerea* is considered to be the second most crucial fungal pathogen

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(Dean *et al.*, 2012) it settles inside the flowers of the strawberry and it may not manifest its presence until the fruit ripens (Rigotti *et al.*, 2002). Anthracnose is a plant disease caused by *Colletotrichum* sp. Due to the severity of this disease, it is considered to be the eighth most important fungal pathogen (Dean *et al.*, 2012). Among them, *Colletotrichum acutatum* infects the strawberry plant without any manifestation of disease (Debode *et al.*, 2009). It can lead to the death of up to 80% of the plants in a nursery, it may also lead to losses in the yields of over 50% in the strawberry cultivation (Debode *et al.*, 2009; Sreenivasaprasad and Talhinhas, 2005; Talhinhas *et al.*, 2005), and to losses of 100% in stored fruit (Dean *et al.*, 2012). The genus *Phytophthora* is an important oomycetes pathogen, and it presents signs of damping-off, chlorosis, dieback, and root rot. *Phytophthora* may cause the death of all plants in a nursery (Prigigallo *et al.*, 2015). *Verticillium* sp. has forms of resistance (microsclerotia) that allow it to remain in the soil for years without a host plant, it can even remain viable in the soil for more than 13 years (Debode *et al.*, 2011). A low concentration of the microsclerotia of the *Verticillium* sp. can lead to a higher level of the disease on the plant (Debode *et al.*, 2011).

The strawberry plant (*Fragaria x ananassa*) has a wide range in susceptibility to various diseases depending on which variant is considered. For this study we chose to investigate three varieties: Honeoye, Rumba and Vibrant, as they belong to the most common for the dessert production in Poland, have different susceptibility to fungal pathogens and are quite resistant climatic conditions (Drobek *et al.*, 2024). However, the Honeoye variety is susceptible to crow rot caused by *Phytophthora cactorum*, red stele caused by *P. fragariae*, verticillium wilt; while is resistant to leaf disease and tolerant to powdery mildew (Beckerman *et al.*, 2022; Colquhoun *et al.*, 2015; Parikka, 2003). Drobek *et al.* (2024) indicated that Vibrant variety was slightly susceptible to *Colletotrichum* sp. and *Phytophthora* sp., but Rumba variety to *Phytophthora* sp. and *Verticillium* sp. phytopathogens.

It is essential to determine the quantity of microsclerotia and oospores, as well as other resting structures, because this is a factor that cannot be underestimated in the field of disease management, with serious consequences. For the detection of latent infections, conventional PCR was used with primers designed to be specific to the ITS1 rDNA region (Debode *et al.*, 2009). Therefore, as a part of the presented study it was decided to design specific primer pairs based on functional genes The most frequently used functional genes (elongation factor 1α, calmodulin, actin, β-tubulin 1 and 2, histone 3 and 4) are highly conserved and they are polymorphic between species and genera (Atallah *et al.*, 2007). Although some PCR methods do not have as high a sensitivity as the qPCR method (Malarczyk *et al.*, 2019), they are a cheap and easy-to-apply approach in the first screening of soil and plants to evaluate the state of their quality.

The research aimed to detect the four important fungal and fungal-like pathogens of strawberry plants, including the design of species/genus-specific primer sets based on functional genes on pure cultures of fungi, as well as on naturally and artificially contaminated environmental samples, such as soil and plant tissues (roots, shoots and fruit). The goal is to develop a straightforward and rapid method for the detection of these potential phytopathogens that guarantees a high degree of efficiency in terms of identification, being able to recognize the disease immediately, and hence organizing the specific plant protection and appropriate treatments to be applied to counter them. For this reason, in our work, we included not only experiments organised in laboratory conditions but also a strawberry field experiment where, on three different strawberry cultivars, different bio-preparations were applied to reduce or eliminate the presence of these four potential phytopathogens from the strawberry plant.

2. MATERIAL AND METHODS

2.1. Potential phytopathogen isolates and environmental samples

2.1.1. Potential phytopathogen isolates

In order to develop a detection method and check its specificity four strains of plant fungal (G669/16 *Botrytis* sp., G171/18 *Colletotrichum* sp., G277/18 *Botrytis* sp., G296/18 *Verticillium* sp.) and one fungal-like (G408/18 *Phytophthora* sp.) pathogens were included in the study. The pure fungal cultures used in the study were derived from the collection of the Laboratory of Environmental and Molecular Microbiology, Institute of Agrophysics, Polish Academy of Sciences (Lublin, Poland). These strains were previously isolated from infested strawberry plants with visible disease symptoms, their phytopathogenicity was confirmed, and they were identified based on the D2 LSU rDNA region through Sanger's sequencing method. The sequences obtained were deposited in the National Centre for Biotechnology Information (NCBI) under the following accession numbers KX639294 (G699/16 *Botrytis* sp.), MT126802 (G171/18 *Colletotrichum* sp.), MT154304 (G277/18 *Botrytis* sp.), MT133320 (G296/18 *Verticillium* sp.) and MT126670 (G408/18 *Phytophthora* sp.) as described in Malarczyk *et al.* (2020).

2.1.2. Environmental samples

Environmental samples were collected from organic plantations of strawberries located in south-eastern Poland. The samples included 2 soil samples (334/19-335/19), 2 roots strawberry samples (333b/19, 336b/19), 2 shoots samples (333/19, 336/19) and 8 samples of strawberry fruit (241/19-248/19). All tested samples were collected from unhealthy strawberry plantations, including soil contaminated by phytopathogens and plants (shoots, roots and fruits) with visible symptoms of diseases. The diagnosis of pathogen infection was performed by combining the symptoms of the plants and microbiological findings, such as culture and PCR results.

2.1.3. Primer design

For each target pathogen, a single primer set was developed using multiple sequence alignments of a representative taxonomic range of fungal sequences downloaded from the

Table 1. List of primers that were newly designed in this study

Phytopathogens	Functional gene	Primer name	Primer[5]	PCR-product	Nº patent[4]
Botrytis cinerea	GAPDH [1]	Btr_GPADH_F	5'- TCCTTGCCGATGGATTGGA -'3	406 bp	P.431989
		Brt_GAPDH_R	5'- ACGCCAATCCTTAGCGGAT -'3		
Colletotrichum sp.	GAPDH [1]	Cll_GAPDH_F	5'- TCATTCCACCTTACCCCTCC -'3	250 bp	P.431991
		Cll_GAPDH_R	5'- GTGGAGTCGTACTTGAGCATG -'3		
Phytophthora sp.	TUB [2]	Pht_490TUB_F	5'- GCCGAYGARGTCATGTGCYTGGATAA -'3	373 bp	P.431992
		Pht_845TUB_R	5'- CGTCCGCGGAACATRCACG -'3		
Verticillium sp.	ACT [3]	Vrt_ACT_F	5'- TAYAGAAGAAGTYGCYGCCCTC -'3	236 bp	P.431993
		Vrt_ACT_R	5'- CCTTGCACATACCCGAACTAC -'3		

[1]GAPDH: glyceraldehyde-3-phosphate dehydrogenase gene, [2]TUB: β-tubulin gene, [3]ACT: actin gene, [4]The primers are protected under Polish Patent Application. [5]The position of the forward and reverse primers for each phytopathogen was described in Figs. S1-S4.

National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov, accessed year: 2018), which were used to select functional gene sequences that were appropriate to the design of the primers. The primers were designed based on the consensus sequences of the functional genes encoding: *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) for *B. cinerea* and *Colletotrichum* sp., *β-tubulin* (TUB) for *Phytophthora* sp., while for *Verticillium* sp. the *actin* (ACT) gene was chosen. In order to design primers for the detection of *Botrytis cinerea*, *Colletotrichum* sp., *Phytophthora* sp. and *Verticillium* sp., we used different quantities of sequences. All of the uploaded sequences were aligned and analysed through the use of MEGA 7 software (accessed year: 2018; Kumar *et al.*, 2016). The primers were placed in consideration of the conserved regions of the selected genes, in the most stable positions with the least and the highest degree of variation, targeting fungal and fungal-like pathogens, and non-target fungi, respectively (Table 1). In the end, the specificity of each newly designed primer was verified using the Basic Local Alignment Search Tool (BLAST) (Camacho *et al.*, 2009) against all of the sequences from the database in NCBI that were obtained in this study.

2.2. DNA extraction

2.2.1. DNA extraction from fungal and fungal-like isolates

The DNA from all individual fungal and fungal-like isolates was extracted after 10 days of growing cultures at 22°C on Potato Dextrose Broth (PDB) following the protocol describes inside Malarczyk *et al.* (2020).

2.2.2. DNA extraction from environmental samples

In the initial step of the study the DNA from naturally infested strawberry fruit was extracted according to the manufacturer's instructions using four different kits: FastDNA™ SPIN kit for Feces (FF) (MP Biomedicals, USA), FastDNA™ kit for Soil (FS) (MP Biomedicals, USA), Plant and Fungi DNA Purification kit (EF) (EURx, Poland), and a Soil DNA Purification kit (ES) (EURx, Poland). Therefore, in the subsequent stages of the research, the environmental DNA (eDNA) from the soil and plants (roots, shoots and fruit) that were collected from naturally infected strawberry plantations, as well as from artificially contaminated samples of soil and fruit, was extracted using two different kits: FastDNA™ SPIN kit for Feces and a Soil DNA Purification kit. The extraction was performed using 0.25 g of plant material or 0.5 g of soil following the manufacturer's instructions after homogenization at 6 m s^{-1} for 40 s using the FastPrep24 instrument.

2.3. PCR assay

2.3.1. Specificity of the designed primers

The validation of the primer's specificity was performed by detecting the four specially selected pathogenic fungi using DNA extracted from pure fungal cultures. Furthermore, we tested the specificity of these primers versus three different fungi that are not recognized as the strawberry phytopathogens: G16/16 *Petriella setifera* (MG594610.1), G132/14 *Neosartorya fischeri* (KM822684.1), and G444/18 *Gnomonia radicicola* (MW175283.1) (Malarczyk *et al.*, 2020; Pertile *et al.*, 2018). The optimized PCR conditions applied in the study for each potential phytopathogen are prepared with REDTaq® ReadyMix™ PCR Reaction mix (Sigma) and presented in Table 2. The quality of the PCR-products obtained was determined electrophoretically in a 2% (w/v, run in SimplySafe, EURx at 85 voltage) agarose gel with the confirmation of PCR-product through an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Moreover, phylogenetic analysis of *B. cinerea* (G276/18 (MW826561); G277/18 (MW826562); G321/18 (MW826563); G322/18 (MW826564); G323/18 (MW826565)), *Colletotrichum* sp. (G171/18 (MW829155); G404/18 (MW829156); G406/18 (MW829157)), *Phytophthora* sp. (G429/18 (MW829178); G430/18 (MW829179); G421/18 (MW829177); G420/18 (MW829176); G419/18 (MW829175); G417/18 (MW829173); G415/18 (MW829172); G413/18 (MW829171); G408/18 (MW829168); G409/18 (MW829169);

Table 2. Description of the primer's concentration and PCR thermoprofile for each fungal and fungal-like pathogen detection

Phytopathogens	Final concentration primer	DNA	Thermoprofile
Botrytis cinerea (G669/16; G277/18)	0.5 µM	3 µl of diluted gDNA (1:10)	94°C for 3 min; 35 cycles at 94°C for 15 s, 50°C for 30 s, 72°C for 45 s; followed by 72°C for 10 min
Colletotrichum sp. (G171/18)	1 µM	2 µl diluted gDNA (1:10)	94°C for 3 min; 5 cycles at 94°C for 15 s, 56°C (touchdown -1°C) for 15 s, 72°C for 15 s; followed by 25 cycles at 94°C for 15 s, 50°C for 15 s, 72°C for 15 s; followed by 72°C for 7 min
Phytophthora sp. (G408/18)	1 µM	2 µl diluted gDNA (1:10)	94°C for 3 min; 35 cycles at 94°C for 15 s, 60°C for 40 s, 72°C for 45 s; followed by 72°C for 10 min
Verticillium sp. (G296/18)	1 µM	2 µl diluted gDNA (1:10)	94°C for 3 min; 5 cycles at 94°C for 15 s, 65°C (touchdown -1°C) for 15 s, 72°C for 15 sec; followed by 25 cycles at 94°C for 15 s, 60°C for 15 s, 72°C for 15 s; followed by 72°C for 7 min

G432/18 (MW829180); G434/18 (MW829181); G439/18 (MW829183); G442/18 (MW829185); G412/18 (MW829170); G437/18 (MW829182); G418/18 (MW829174); G441/18 (MW829184)), *Verticillium* sp. (G293/18 (MW829158); G294/18 (MW829159); G296/18 (MW829160); G297/18 (MW829161); G298/18 (MW829162); G302/18 (MW829163); G328/18 (MW829164); G330/18 (MW829165); G335/18 (MW829166); G344/18 (MW829167)) and *C. acutatum* (G162/18) (PV013547) sequences based on the specific functional genes GAPDH, GAPDH, TUB, ACT, GAPDH, respectively. Phylogenetic trees for each phytopathogen separately were prepared using obtained sequences from pure strains and environmental samples sequences originating from National Center for Biotechnology Information (NCBI). The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model with rate gamma distributed plus invariant sites (*B. cinerea*), the Maximum Likelihood method and Tamura-Nei model with rate gamma distributed (*Colletotrichum* sp., *C. acutatum*), the Maximum Likelihood method and Tamura-Nei model with invariant sites (*Phytophthora* sp.) and the Maximum Likelihood method and Tamura-Nei model with rate gamma distributed (*Verticillium* sp.). The Bootstrap method with 4000 number of Bootstrap replications was applied using MEGA 7 software (Tamura *et al.*, 2016). All accession numbers of the sequences obtained from the sequencing process are presented in Tables S1-S3.

2.3.2. Potential phytopathogen detection in naturally infected and artificially contaminated environmental samples

The developed PCR assays were validated on various types of environmental samples in several steps. Initially, the study included eight naturally infested samples of strawberry fruit that were tested using the different commercial kits described above. The EF and ES kits were also used in all subsequent steps of the research for the pathogenic fungi of the tested plants. Subsequently, in order to evaluate the influence of the incubation time on pathogen growth and detection effectiveness, selected naturally infested strawberry fruit (241/19, 245/19, 246/19) were incubated at 24°C in Potato Dextrose Broth (PDB) for 24, 48 and 72 h. Next, an assessment of the plant pathogens present in the naturally infested soil (334/19; 335/19), shoots (333/19; 336/19), and roots (333b; 336b/19) of strawberry plants was performed.

Moreover, non-contaminated soil and fruits from healthy organic strawberry plantation were collected to study particular artificial contaminations of samples by selected potential phytopathogens. Before experiment soil and fruits were sterilised in autoclave and then they were artificially contaminated by inoculation with fungal spores. First, the soil was divided into sub-samples which were inoculated with the following spore concentrations: 500-1 000-5 000 and 10 000 spores per g of soil, and analysis for each of the four pathogens was performed following 24- and 48-h of incubation at 24°C. The next step of experiment included inoculation of sterile soil and strawberry fruits with by the following fungal spore concentrations: 100 000-10 000-1 000 and 100 spores per g of soil/fruit. In this experiment, the analysis for pathogens was performed directly after the inoculation of the soil and strawberry fruit and following an incubation period of 72 h at 24°C. Each artificially contaminated sample was infected with a mixture of spore concentrations from each pathogen as described by Malarczyk *et al.* (2020).

2.4. Field experiment

2.4.1. Field principal characteristics

The field experiment was performed at the Institute of Soil Science and Plant Cultivation State Research Institute in Puławy and located in the Agricultural Experimental Station in Grabów in Mazowieckie region (51°21′17.1″ N 21°39′14.0″ E, Poland). The experiment was set up on Luvisols on a gray-brown podzolic soil made of strong loamy sands on light loam, in a split-block design in 4 replication as described Nakielska *et al.* (2024). The area of each plot of strawberries was 16 m^2. In each plot 48 strawberry plants were planted at 30 x 100 cm spacing at plant density 30 thousand plants per hectare. The experiment was set up using three different strawberry cultivars:

Honeoye, Vibrant, and Rumba, belonging to the most popular for the dessert production in Poland, having various resistance to fungal pathogens and being quite resistant to weather conditions. The experimental area was covered with fleece to reduce evaporation and weed growth, and the field experiment was conducted with and without irrigation conditions. The field with irrigation system was irrigated 6 times during the growing season with 6 000-7 000 l of water.

The field experiment included seven experimental variants with 5 combinations of biopreparations with beneficial microorganisms and plant extracts which were tested in comparison to 2 control treatments as described Drobek *et al.* (2024). Composition of biopreparations and control treatment was described by Drobek *et al.* (2024) and Nakielska *et al.* (2024). Briefly, the two controls were set up, one with carrier (Control I) and second (Control II) without the carrier on which each biopreparation was developed. The carrier was composed of nettle extract, horsetail extract, calendula extract, liquid humic acids, Vinasa-yeast culture effluent, bran, dry humic acids, mustard, rapeseed oil. Control without carriers was only sprayed with water. Five experimental variants with biopreparations included: I. (P1) *Bacillus subtilis* AF75AB2 and *Bacillus* sp. Sp115AD, on a carrier consists of plant extracts (nettle, horsetail, calendula), humic acids in liquid formulation and (P3) *Bacillus* sp. AF75BC and *Bacillus subtilis* AF75AB2, on a carrier consists of wheat bran, dry humic acids, mustard, rapeseed oil, clove oil in a pellet formulation; II. (P2) *Bacillus* sp. Sp116AC*, *Bacillus* sp. Sp115AD, humic acids, yeast culture effluent in liquid formulation and (P3); III. (P1) + (P2) + (P3); IV. (P1) + (P2); V. (P3). The biopreparation combinations were designed as biotechnological solutions and agricultural management technologies to support strawberry plants growth and vitality with ready-to-use liquid (P1+P2), solid (P3) and liquid-solid (P1+P3; P2+P3; P1+P2+P3) formulations. The solid biopreparation (P3) and carrier (Control I) were applied as pellet form under plant roots, only once in May during growing season, while liquid biopreparations (P1, P2) and water in control (Control II) were applied twice in May and June during growing season using a tractor sprayer with a Fragaria type boom with spraying from above and from sides (Nakielska *et al.*, 2024). The solid biopreparation was applied at a dose of 10 g per plant when the concentration of bacteria was at the level of 108 cfu g^{-1} of biopreparation, which corresponds to 1 g per plant when the bacterial concentration is 10^9 cfu g^{-1} of biopreparation. Liquid biopreparation was applied as a solution of powdered inoculum at a dose of 35 kg when bacterial concentration was at the level of 10^8 cfu g^{-1} of biopreparation diluted in 700 l of water per ha, corresponding to 3.5 kg per ha in 700 l of water when bacterial concentration was at 10^9 cfu per g of biopreparation. The bacteria used for biopreparation originated from the SYMBIOBANK collection of the Research Institute of Horticulture in Skierniewice (Poland) and they were described by Trzciński *et al.* (2021). Metabolic properties, activity and interactions between bacterial strains included as component of biopreparations were presented in patent applications (P.445011, 2023; P.445051, 2023; P.445052, 2023; P.445053, 2023; P.445054, 2023). The soil, leaves and fruit were collected the 9th June 2021 and immediately conserved on ice. In total, 126 samples of rhizosphere soil (42 samples), leaves (42 samples) and fruits (42 samples) were collected. Samples were collected randomly in three replications from each plot, wherein one replication was mixed from 5 separate strawberry plants in order to obtain an average sample (~200 g) and storage at -20°C until the DNA extraction by Soil DNA Purification kit (EURx, Poland) according to the manufacturer's protocol was performed.

2.4.2. Detection of the presence of the potential phytopathogens in soil, leaves and fruit of strawberry plantation

For the field experiment we used the same specific primer (*Botrytis cinerea*, *Phytophthora* sp., and *Verticillium* sp.) previously tested in laboratory conditions and furthermore, we used a specific primer for *Colletotrichum acutatum*.

The pair of primer was drawn based on the sequence of the functional gene *glyceralde-hyde-3-phosphate dehydrogenase* (GAPDH). The sequence of the primer CactGAPDH_F (5'- ACGATAACACCAGCTTCGTCGAT-'3) and CactGAPDH_R (5'- TCTGCATGAYTGKGTCACGTCG-'3) (Fig. S5). For this primer, we utilised 4 µl of diluted (1:10) template DNA and optimised a proper thermoprofile written below: 94°C for 3 min; following 5 cycles at 94°C for 15 s, 63.5°C (touchdown -1°C for each cycle) for 15 s, 72°C for 15 s; following 45 cycles at 94°C for 15 s, 58.5°C for 15 s, 72°C for 15 s; following 72°C for 7 min. The utilisation of this primer, we obtained a PCR-product of 149 bp.

All information concerning specific primers used are included in the Polish Patent Applications: P.431989, P.431991, P.431992, P.431993 and P.445011.

2.5. Statistical analysis

All the results from the PCR analyses (obtained from the environmental samples) were transformed in presence and absence table in function of the existence or lack of the PCR-product. The table was used to create a heatmap by the Euclidean matrix, and for the cluster analysis, a dendrogram was calculated with the Ward method. All analyses were performed using R software (version 4.3.1; packages: ComplexHeatmap, *circlize*) (Gu, 2022; Gu *et al.*, 2016, 2014).

3. RESULTS

3.1. Fungal and fungal-like isolates and the specificity of the designed primers

The analysis of the four primer pairs on the pure culture of each pathogen (Fig. 1) confirmed that all of the designed primers are genus/species-specific. Each primer amplified the correct PCR-product for each specific gene and organism. By reviewing the phylogenetic trees, we may observe that the sequence obtained from the utilisation of the Brt_GAPDH_F and Brt_GAPDH_R primers was clustered together with the sequences of *Botrytis cinerea* and only one

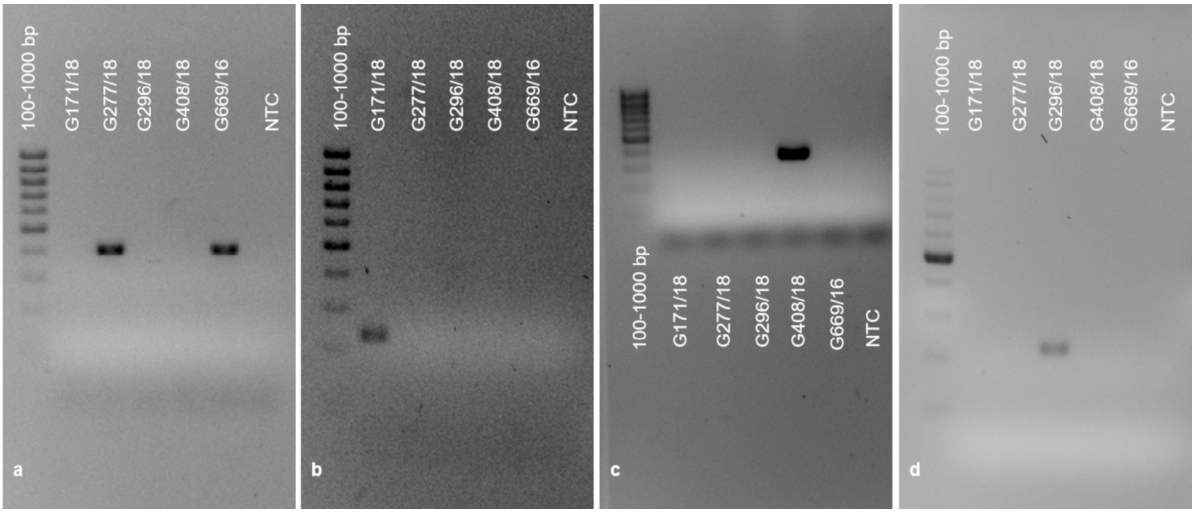

Fig. 1. PCR amplification using *B. cinerea* (a), *Colletotrichum* sp. (b), *Phytophthora* sp. (c), and *Verticillium* sp. (d) specific primers in pure culture. Gel agarose 2% with 100-1 000 bp ladder. NTC means non-template control. The tested strains were the following: *Colletotrichum* sp. (G171/18), *Botrytis* sp. (G277/18, G669/16), *Verticillum* sp. (G296/18), and *Phytophthora* sp. (G408/18).

sequence of *B. pseudocinerea*, completely separated from the other clusters formed by other fungi belonging to the same Sclerotiniaceae family (Fig. S7). For the *Colletotrichum* sp. (Fig. S8), a complete separation between the *Colletotrichum* sp. and the other species belonging to the Sordariomycetes class was observed. Furthermore, our three pure fungal cultures were clustered together with other database sequences of the GAPDH gene belonging to C. *acutatum*. In the case of *Phytophthora* sp. (Fig. S9), a division between *Phytophthora* sp. and *Nothophytophthora* sp. versus the other species belong to the Peronosporaceae family was obtained. The sequence of our pure culture strains were clustered inside the group of *P. cactorum*, which is main strawberry pathogen. For *Verticillium* sp. (Fig. S10), we obtained two distinct clusters, one was composed of the other species belong to the Sordariomycetes class, whereas the other cluster was composed of *Verticillium* sp. Our sequences were clustered inside the group of *Verticillium* sp. and particularly inside the group of *V. dahliae*. From these phylogenetic trees, we may observe the specificity of the designed primers presented in this paper. Furthermore, the specificity of the primers versus other fungal genera was analysed, and the same PCR product as for the specific detection was not observed when the designed primers were used (Fig. S11).

3.2. Detection of fungal and fungal-like pathogens in naturally infected environmental samples

In naturally infested strawberry fruits compared the results between *Botrytis cinerea* and *Phytophthora* sp., we observed the creation of three different clustering groups (Fig. S12). The first group was composed of those samples that showed the absence or presence of the two analysed potential phytopathogenic fungi. Whereas, inside the group two, they underlined the samples in which they had identified the presence of *B. cinerea*, and in the group four, we found the samples that were identified as having the presence of *B. cinerea* and *Phytophthora* sp. or *Phytophthora* sp. alone. If we looked at the type of DNA extraction kit, *B. cinerea* was detected in almost all of the analysed samples extracted using the FastDNA™ SPIN kit for Feces (FF) (241/19, 242/19, 243/19, 244/19, 245/19, 246/19, 247/19, 248/19) (Fig. S19). In the case of the *Phytophthora* sp., the detection indicated the presence of this oomycete pathogen in a few samples (Fig. S19), where the amount of the PCR-product was very high. In the DNA extracted using the Plant and Fungi DNA Purification kit (EF), the presence of *B. cinerea* and *Phytophthora* sp. was not detected (Fig. S12). Also, *Colletotrichum* sp. and *Verticillium* sp. were detected in a few samples (Fig. S13). Analysing the results obtained for these two fungal pathogens, we saw a clear separation into three clusters: one was presented by those samples that did not show the presence of both analysed pathogens while the other two groups showed the presence of either *Colletotrichum* sp. or *Phytophthora* sp.. For all four pathogens, it was observed that the FF kit was the best one for environmental DNA (eDNA) extraction from naturally infested strawberries. Furthermore, we aimed to analyse the presence of potential phytopathogens after incubation at 24°C for 24, 72, and 48 h in three different samples of naturally contaminated strawberries (Fig. 2). By analysing the results, we could observe the division of the samples into four groups depending on the presence/absence of the potential phytopathogens inside strawberries incubated at 24°C. The first group contained the samples that recorded the presence of *Colletotrichum* sp. *Phytophthora* sp.; the presence of *B. cinerea, Colletotrichum* sp. and *Verticillium* sp. were grouped in the third group, while in the fourth group, we found the two samples (241/19 after 24 and 48 h incubation) in which we found the simultaneous presence of all four potential phytopathogens. *B. cinerea* was

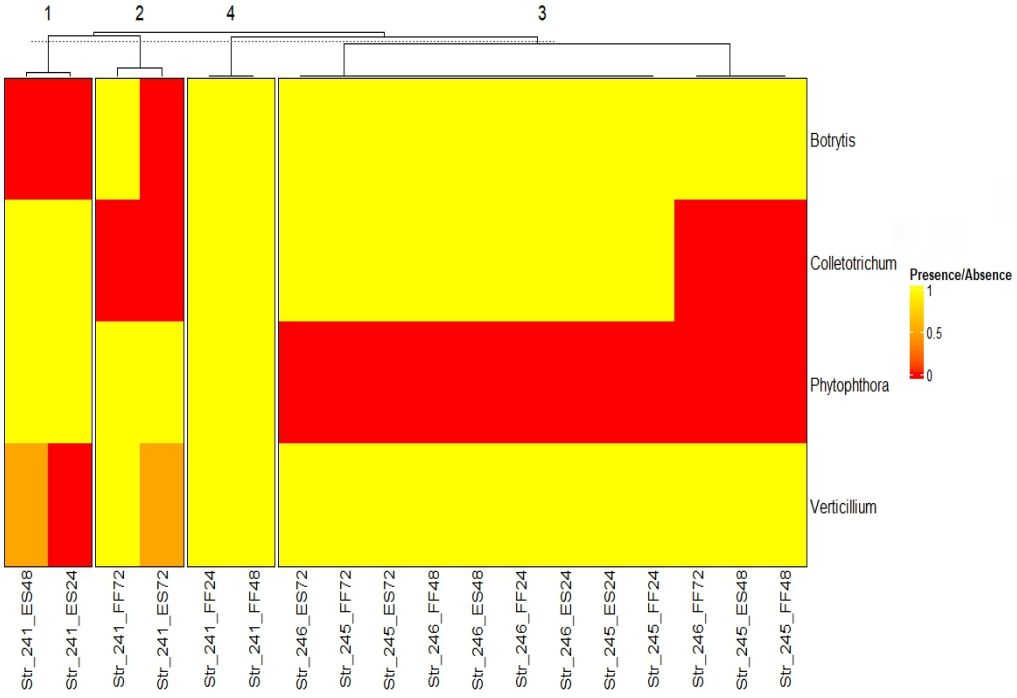

Fig. 2. Heatmaps about the presence/absence of *B. cinerea* (Botrytis), *Colletotrichum* sp. (Colletotrichum), *Phytophthora* sp. (Phytophthora), and *Verticillium* sp. (Verticillium) in strawberry fruit (Str) naturally infected incubated at 24°C for 24, 48, and 72 h using 2 different kits of DNA extraction (FastDNA™ SPIN kit for Feces (FF), and Soil DNA Purification kit (ES)). 241 and 246 – fruit samples with symptoms of the pathogens presence, 245 – healthy fruit sample without visible disease symptoms.

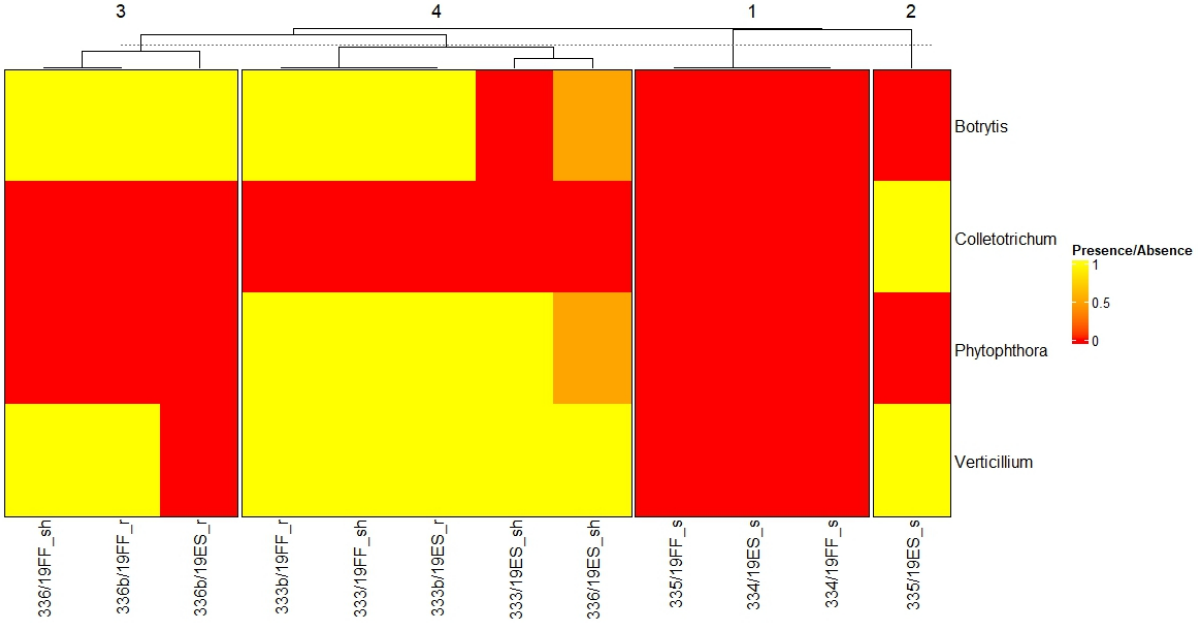

Fig. 3. Heatmaps about the presence/absence of *B. cinerea* (Botrytis), *Colletotrichum* sp. (Colletotrichum), *Phytophthora* sp. (Phytophthora), and *Verticillium* sp. (Verticillium) in soil (s) (334/19, 335/19), shoots (sh) (333/19. 336/19) and roots (r) (333b/19, 336b/19) from strawberry plantation naturally infected using two different kits of DNA extraction extraction (FastDNA™ SPIN kit for Feces (FF), and Soil DNA Purification kit (ES)).

detected in all three samples (241/19, 245/19, 246/19) after the incubation time (Fig. S21a), whereas *Colletotrichum* sp. was only noted in two samples (Fig. S21b). By contrast, *Phytophthora* sp. was found but only in one sample (Fig. S21c), and in particular, the intensity of the PCR-product was very intense on the eDNA extracted using the FastPrep kit. The presence of the *Verticillium* sp. was detected in all tested samples (Fig. S21d). By analysing samples from plants, the soil and the roots of a strawberry plantation, we observed the detection of all four pathogens (Fig. 3) and they were subdivided in four groups. The first group was represented by the samples, which were analysed for potential phytopathogens, but were not detected using the FF and ES kits. The next group included the only soil samples extracted using the ES kit, detecting the presence of *Colletotrichum* sp. and *Verticillium* sp. While in the third and fourth groups, the shoot and root samples were grouped according to the detection of the presence of *Botrytis cinerea*, *Phytophthora* sp. and *Verticillium* sp. Through this analysis, the presence of *B. cinerea* was revealed in the sample 333/19 and 336/19 using the FF kit, whereas *Verticillium* sp. was found in the sample 333/19 and 336/19 extracted with FF and ES kits. By contrast, *Colletotrichum* sp. was only found in the soil. A different situation was observed for *Phytophthora* sp., it was only detected in the shoots and roots. It is worth noting that through this method *Verticillium* sp. was detected in all samples and without incubation.

3.3. Detection of fungal and fungal-like pathogens in artificially contaminated environmental samples

When we analysed the artificially contaminated soil samples (I-IV_ES/FF_24/48) at certain spore concentrations (Fig. S14), we immediately observed that in the first group were all the samples contaminated with the low spore concentrations (500 and 1 000 spores g^{-1} of soil) extracted from both of the two kits used (after a 24 and 48 h incubation) which did not report a detection of the presence of either of the four potential phytopathogens or few presence of *Colletotrichum* sp. (III_ES_24, IV_FF_24). In the second group, on the other hand, we grouped the samples that identified the presence of *Verticillium* sp. only (IIES_24). In particular, we also found samples contaminated with 1,000 spores, extracted using the Soil DNA Purification (ES) kit, after 24 h of incubation. In the penultimate group, we found those samples that showed the presence of *Verticillium* sp. and a few samples with the presence of *Colletotrichum* sp. and *B. cinerea* (IVFF_24, VIFF_48, IIIFF_48, IIIFF_24, IVES_48, IIIES_48, IIIES_24, IVES_24). We observed that the samples in this group were mostly artificially contaminated with 5 000 and 10 000 spores. In the last group were grouped the samples that led to the detection of all potential phytopathogens except of *Phytophthora* sp. and *Colletotrichum* sp. When the soil was artificially contaminated with 500 to 10 000 spores g^{-1} of soil and the samples were incubated at 24°C, only the *Phytophthora* sp. was not detected (IES_24, IIFF_24). *B. cinerea* was detected in one sample contaminated with 1 000 and 10 000 spores after 48- and 24-h of incubation (IIES_48, IVFF_24). The "no robust" detection of *Colletotrichum* sp. was observed after 24- and 48-h of incubation for the higher concentration of spores (5 000 and 10 000 spores g^{-1} of soil). This result confirms that the detection limit for this pathogenic fungus is 5 000 spores g^{-1} of soil. For the *Verticillium* sp., the detection was observed with the medium spores concentration (1 000 spores g^{-1} of soil) after 24 h of incubation, and in particular (Fig. S23). When artificially contaminated soil and strawberry samples, with spore concentrations ranging from 100 to 100 000 spores g^{-1} of environmental sample, were incubated at 24°C for 0 and 72 h and all four pathogens were detected inside the analysed samples (Figs 4 and S15). Analysing the soil samples extracted through ES kit (Fig. S15a), we can observe that in the first group we found more the samples contaminated with low spore concentrations (100 and 1 000 spores) that did not lead to the detection of the presence of the four potential phytopathogens analysed, except for the sample contaminated with 100 spores g^{-1} of soil after 72 h of incubation that led to the detection of only the presence of *Verticillium* sp. In the next group, however, we found the sample contaminated with 100 spores, which, after a 72-h incubation, led to the sole detection of the presence of *B. cinerea*. Within the third group, we located only the sample contaminated with 10 000 and 100 000 spores after a 72-h incubation, leading to the identification of the presence of *B. cinerea* and *Colletotrichum* sp. In the last group, samples that reported the presence of all potential phytopathogens, except *Phytophthora* sp., were grouped together. In contrast, the same soil samples extracted with the FF kit presented completely different results (Fig. 4a). In the first group, two samples contaminated with 100 and 1,000 spores incubated for 72 h presented only the presence of *Verticillium* sp. In the next group, we observed the sample contaminated with 100 spores, which resulted in the detection of the presence of *B. cinerea* and *Verticillium* sp. Within the penultimate group, we observed that the sample that was not incubated was contaminated with 1 000 spores, leading to the detection of *B. cinerea* and *Colletotrichum* sp., while the sample that was contaminated with 100 000 spores, which led to the detection of only the presence of *Colletotrichum* sp.. In the last group were all those samples that presented the simultaneous presence of almost all the potential phytopathogens considered in this study. DNA extracted from artificially contaminated strawberries using the ES kit (Fig. S15b) led to the simple detection of the presence of *Phytophthora* sp. in samples contaminated with 1 000-100 000 spores; while the results obtained using the FF kit (Fig. 4b) demonstrated that only in the contaminated sample with the highest concentration of spores did the simultaneous detection of all four potential phytopathogens analysed occur. By analysing the eDNA extracted using the ES kit, all pathogens were detected in the soil samples, especially after the incubation period, except *Phytophthora* sp. which was detected only after incubation in the strawberry samples and it was the only pathogen detected inside the strawberry samples. *Botrytis cinerea* was also detected in all strawberry samples with or without an incubation period. In the case of

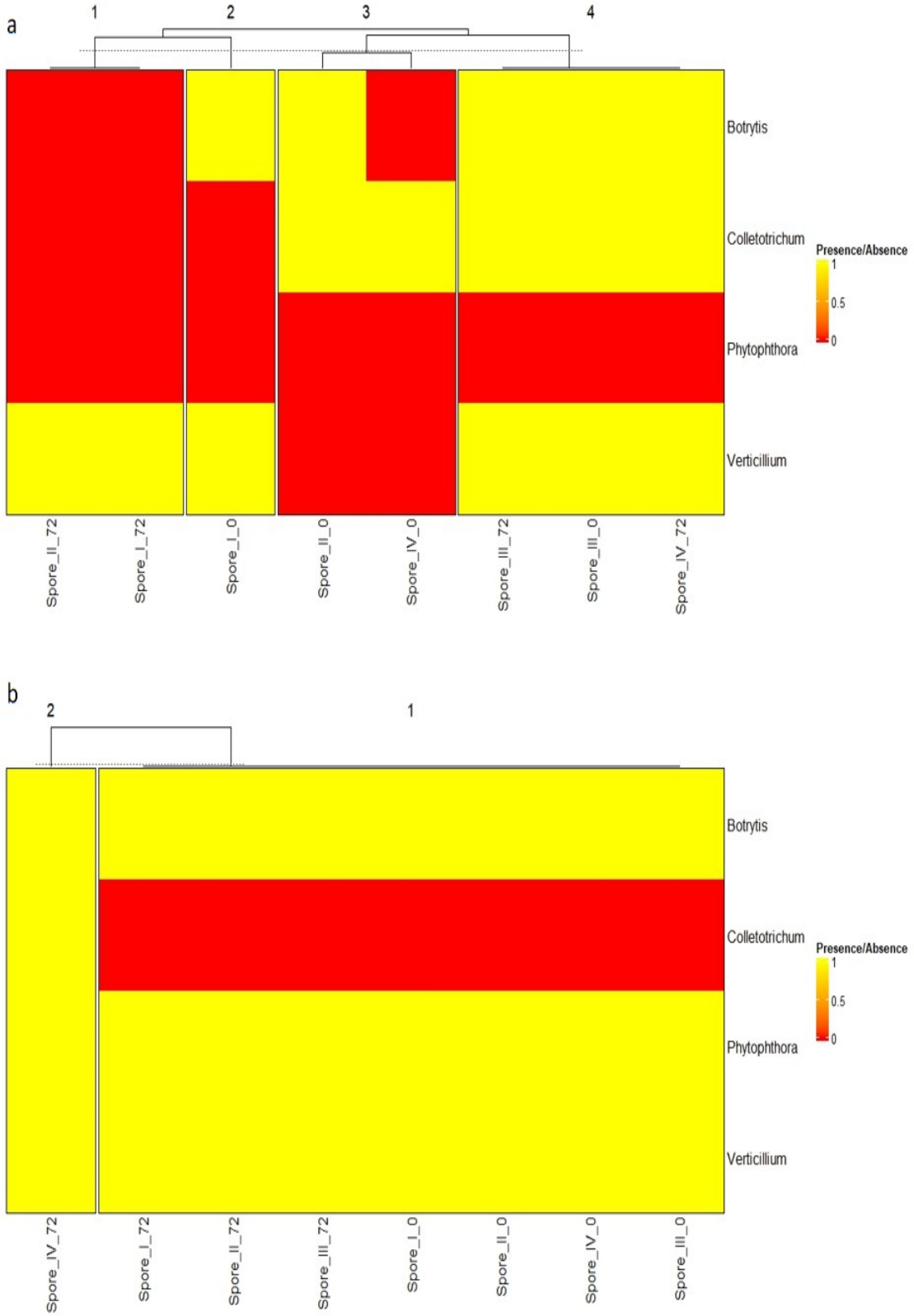

Fig. 4. Heatmaps about the presence/absence of *B. cinerea* (Botrytis), *Colletotrichum* sp. (Colletotrichum), *Phytophthora* sp. (Phytophthora), and *Verticillium* sp. (Verticillium) in soil (a) and strawberry fruits (b) artificial contaminated samples incubated at 24°C for 0 and 72 h after DNA extraction with the FastDNA™ SPIN kit for Feces. Explanations: Spore_I – addition of 100 spores per gram of soil/fruits; Spore_II – addition of 1000 spores per gram of soil/fruits; Spore_III – addition of 10000 spores per gram of soil/fruits; Spore_IV – addition of 100000 spores per gram of soil/fruits.

Colletotrichum sp., detection was observed in strawberry samples with spore concentrations of 100 000 spores g^{-1} of fruit after incubation. In contrast, for soil samples, the detection of the PCR-product was confirmed in samples with medium spores concentrations (10 000 – 10 000 spores g^{-1} of soil) and after incubation in samples contaminated with high spore concentrations (100 000 – 10 000 spores g^{-1} of soil). *Phytophthora* sp. was detected in all strawberry samples at all spore concentrations, with or without an incubation period. The last fungal pathogen, *Verticillium* sp., was detected in all strawberry samples with or without an incubation period, whereas in the soil samples, it was only found in samples with high spore concentrations (100 000 spores g^{-1} of soil) after the incubation period.

3.4. Effect of biopreparation on the presence of the four potential phytopathogens

Testing the primer specific for *Colletotrichum acutatum* in pure culture and through the Sanger sequencing, confirm the specificity of the our primer (Figs S16-S17). Through the phylogenetic tree, we could observe a clear separation at the family level between different inserted fungi belonging or not to the same family of *Colletotrichum* sp.. Furthermore, our strain, identified by our primers as *C. acutatum*, was inserted within the cluster of *C. acutatum* and *Glomerella acutata* (since it is the teleomorphic phase of *C. acutatum*) (Peres *et al.*, 2005).

By analysing the presence/absence of these four potential phytopathogens, we observed a different response after the application of the different bio-treatments. From the rhizosphere of the Honeoye cultivar (Fig. 5a), we could observe a not strong response in the reduction of potential phytopathogens after the application of the analysed bio-treatments. Analysing the irrigated system in more detail, the control (Hon_wat_A/B) showed a total absence of *Botrytis cinerea* and *Colletotrichum acutatum*, while there was an almost zero and medium presence of *Verticillium* sp. and *Phytophthora* sp., respectively. The P1+P2 treatment reduced the presence of *Phytophthora* sp., while the presence of *C. acutatum* increased compared to the control. On the contrary, in the non-irrigated system, we noticed that the P3 and P3+P1+P2 treatments led to a decrease in the presence of *Phytophthora* sp. and *Verticillium* sp. and to increase in the presence of *C. acutatum*. For the Vibrant cultivar (Fig. 5b), we observed the absence of *B. cinerea* and *C. acutatum*, a medium presence of *Phytophthora* sp. and a strong presence of *Verticillium* in the irrigated system. For this cultivar, the P1+P2 bio-treatment was effective, as it reduced the presence of *Verticillium* sp.. In the case of the non-irrigated system, the control recorded a slight and medium presence of *Phytophthora* sp. and *Verticillium* sp., respectively. In this case, no bio-preparation led to a reduction in the presence of the potential phytopathogens. For the last analysed cultivar, Rumba (Fig. 5c), we saw that in both analysed systems, the bio-preparations that we created did not achieve a substantial reduction in the presence of *Verticillium* sp. and especially in the non-irrigated system the treatments have not shown any effectiveness. Globally analysing the results obtained from the rhizosphere, we can observe a clear response to the application of bio-preparations, depending on the system and cultivar.

By analysing the results obtained from the leaves of the different cultivars, we found different responses specifically depending on the application of the bio-preparations (Fig. 6), Starting with the Honeoye (Fig. 6a), we could say that the bio-preparations applications did not lead to a better situation than what occurred in the control. As far as we could observe, for the irrigated system compared to the control, only the P1+P2 treatment resulted in a reduction in *Phytophthora* sp. and, simultaneously, an increase in the presence of *C. acutatum*. While for the non-irrigated system, we could see that two treatments (P3 and P3+P1+P2) presented a slight increase in the presence of *C. acutatum* and *Verticillium* sp. As we noted for the previous cultivar, for Vibrant (Fig. 6b), no treatment analysed had an effect on the total or almost total absence of *Verticillium* sp. By observing the irrigated systems, the P3+P1+P2 treatment reduced in the presence of *Phytophthora* sp. compared to the control. For the non-irrigated system the control presented a slight presence of *Phytophthora* sp. and a medium presence of *Verticillium* sp., with the consequence that no treatment analysed improved the absence of these four potential phytopathogens, only the P3+P1+P2 treatment presented a situation equal to the control. Observing the leaves collected from the Rumba cultivar (Fig. 6c), we observed a strong presence of *Phytophthora* sp. and *Verticillium* sp. In the irrigated system, we observed how the treatments of P3 and P3+P1+P2 led to an incentive reduction in the presence of *Phytophthora* sp., whereas for the non-irrigated system, a slight decrease in the presence of *Phytophthora* sp. and *Verticillium* sp., was observed in P3+P1. Overall, the results obtained from the leaves showed that the analysed treatments in this study did not have a large impact as observed in the soil, especially towards *Phytophthora* sp. and *Verticillium* sp.

Finally, we analysed the fruit for each cultivar (Fig. S18) and they led to entirely different results from those obtained for the soil and leaves. The Honeoye cultivar (Fig. S18a) in the irrigated system led to a substantial reduction in the presence of the four potential phytopathogens when compared to the control (strong presence of *C. acutatum* and *Verticillium* sp.), and for the non-irrigated system, both the control and the analysed treatments showed zero presence of all examined pathogens. We observed almost the same results for the Vibrant cultivar (Fig. S18b), in fact all the treatments, involved in both analysed system, which presented a substantial reduction (practically zero) of this potential phytopathogens. For the last cultivar taken into consideration, Rumba (Fig. S18c), in the irrigated system the control presented a slight presence of *Phytophthora* sp. and *Verticillium* sp., with the consequent total reduction of them after the application of the following treatments P3, P3+P1, and P3+P1+P2. For the non-irrigated system, we recorded a slight presence of *C. acutatum* in the control, which, with the application of P3+P1 and P1+P2, led to the total absence of these pathogens in the fruit. Overall, we can say that the foliar and soil application of the different

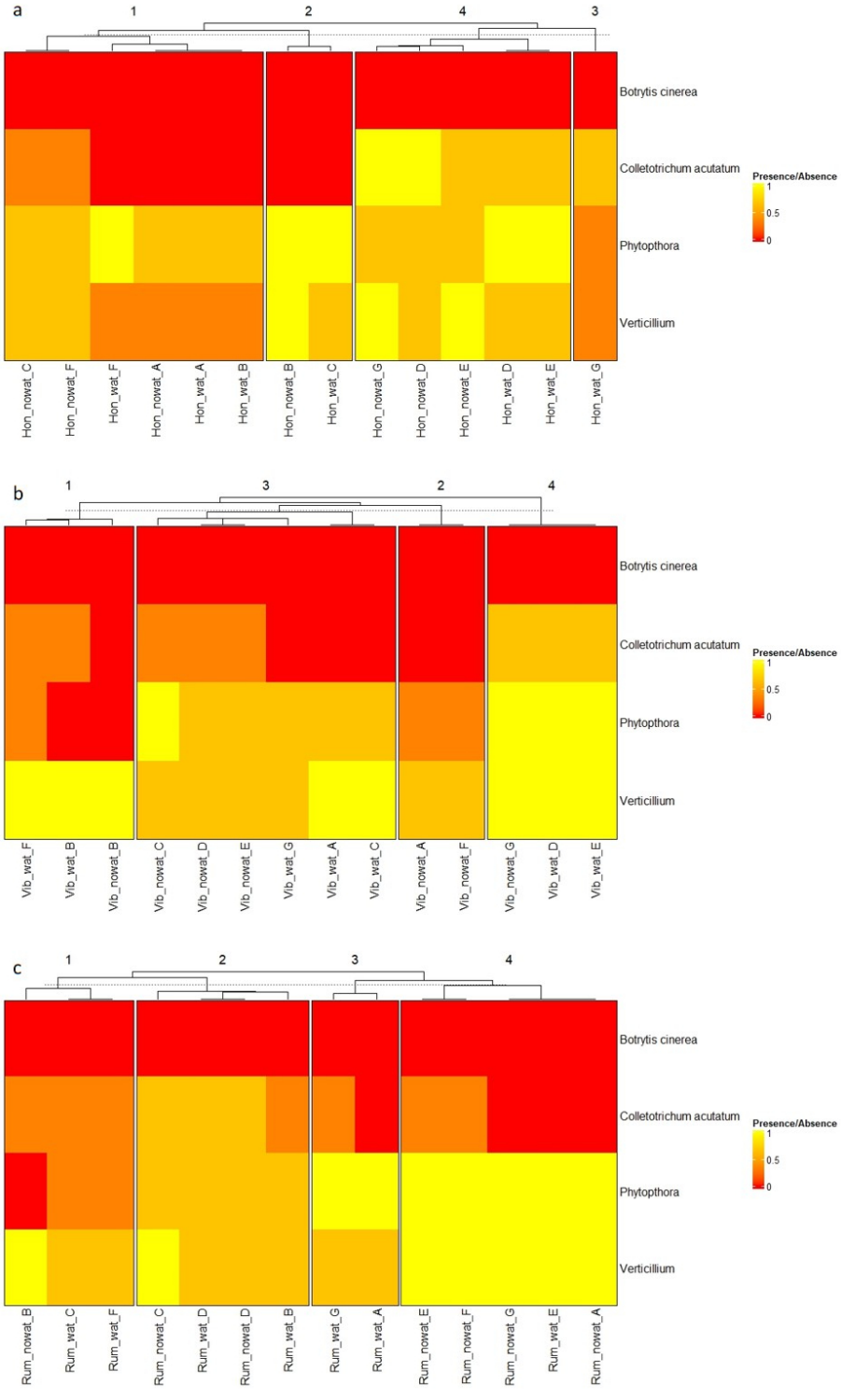

Fig. 5. Heatmaps about the presence/absence of *B. cinerea* (Botrytis cinerea), *Colletotrichum acutatum* (Colletotrichum acutatum), *Phytophthora* sp. (Phytophthora), and *Verticillium* sp. (Verticillium) in rhizosphere soil from field experiment for Honeoye (Hon) (a), Vibrant (Vib) (b), and Rumba (Rum) (c). Explanations: wat – plots with irrigation/watering;nowat – plots without irrigation/watering; A – Control spraying only with water without biopreparations and without carrier (Contron I); B – Control without biopreparations but with carrier (Control II), C – P3 *Bacillus* sp. AF75BC and *Bacillus subtilis* AF75AB2, on a carrier consists of wheat bran, dry humic acids, mustard, rapeseed oil, clove oil in a pellet formulation; D – P3+P1 *Bacillus subtilis* AF75AB2 and *Bacillus* sp. Sp115AD, on a carrier consists of plant extracts (nettle, horsetail, calendula), humic acids in liquid formulation; E – P3+P2 *Bacillus* sp. Sp116AC*, *Bacillus* sp. Sp115AD, humic acids, yeast culture effluent in liquid formulation; F – P3+P1+P2; G – P1+P2.

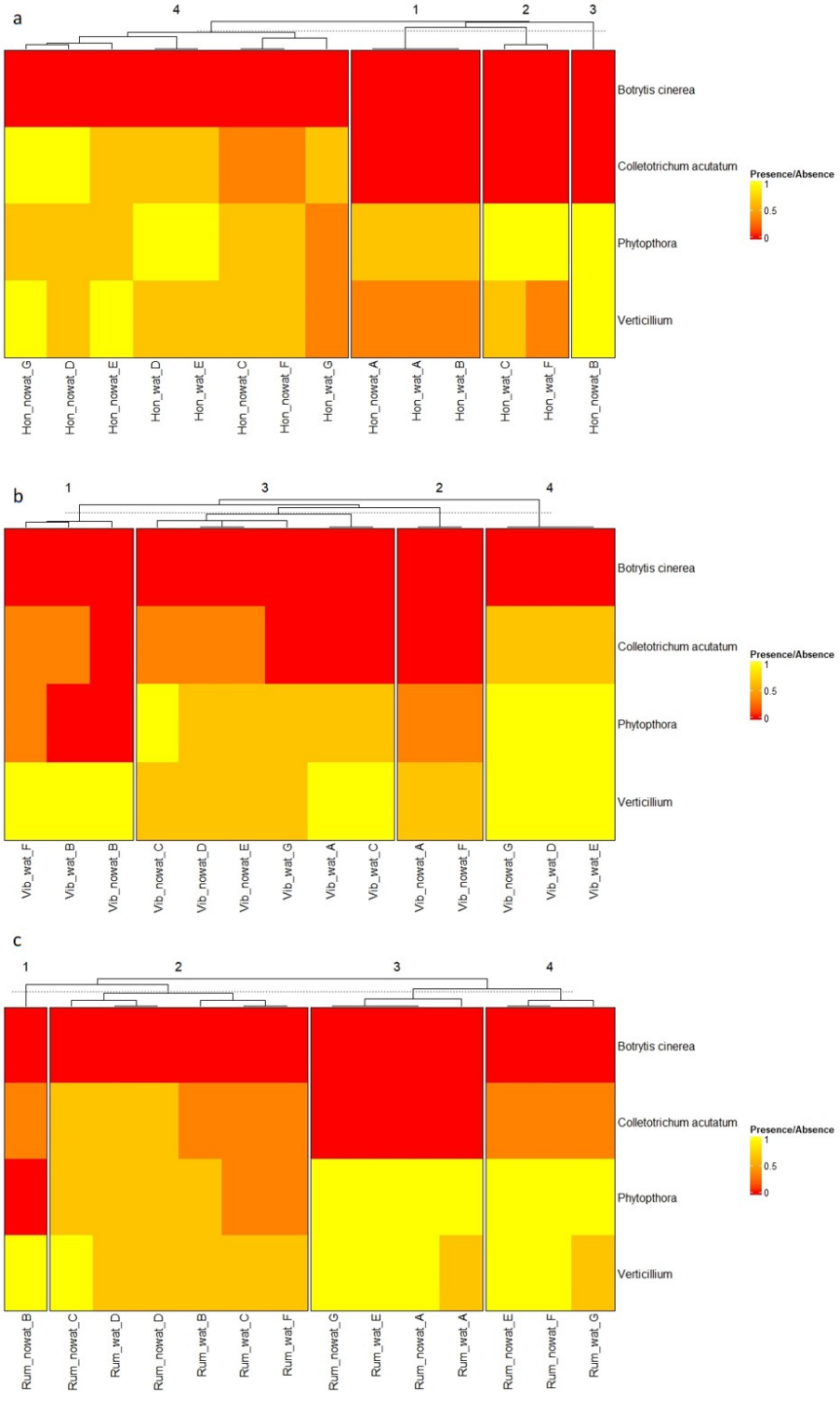

Fig. 6. Heatmaps about the presence/absence of *B. cinerea* (*Botrytis cinerea*), *Colletotrichum acutatum* (*Colletotrichum acutatum*), *Phytophthora* sp. (*Phytophthora*), and *Verticillium* sp. (*Verticillium*) in leaves from field experiment for Honeoye (Hon) (a), Vibrant (Vib) (b), and Rumba (Rum) (c). Explanations: wat – plots with irrigation/watering;nowat – plots without irrigation/watering; A – Control spraying only with water without biopreparations and without carrier (Contron I); B – Control without biopreparations but with carrier (Control II), C – P3 *Bacillus* sp. AF75BC and *Bacillus subtilis* AF75AB2, on a carrier consists of wheat bran, dry humic acids, mustard, rapeseed oil, clove oil in a pellet formulation; D – P3+P1 *Bacillus subtilis* AF75AB2 and *Bacillus* sp. Sp115AD, on a carrier consists of plant extracts (nettle, horsetail, calendula), humic acids in liquid formulation; E – P3+P2 *Bacillus* sp. Sp116AC*, *Bacillus* sp. Sp115AD, humic acids, yeast culture effluent in liquid formulation; F – P3+P1+P2; G – P1+P2.

analysed bio-preparations in this work led to a complete reduction of the presence of this potential phytopathogen on the strawberry fruit.

4. DISCUSSION

This study aims to design genus- and species-specific primers to establish a rapid and efficient method for detecting the presence of the most economically important fungal pathogens of strawberry plantations. This was the first study that included agricultural and horticultural fungal and fungal-like pathogens detected in raw materials and environmental samples. It is worth mentioning that the functional genes (ACT, GAPDH, and TUB) selected for the study are present in the fungus with only one copy of each gene (Black *et al.*, 2013; Duressa *et al.*, 2012; Lima *et al.*, 2009; Nahimana *et al.*, 2000). Thus, they are essential for quantification due to the ability to monitor the fungal metabolic activities to which these functional genes are linked.

By analysing each type of sample, *B. cinerea* was detected in all samples of naturally contaminated strawberry (Fig. S12). Whereas in the plant and soil samples, the pathogen causing grey mould was only found in the plants and roots (Fig. 3). In the strawberry sample without an incubation period the presence of *B. cinerea* was not detected (Fig. S12), but when it was incubated at 24-, 48-, and 72 h the presence of the fungal pathogen was revealed (Fig. 2). This approach can be useful when the low concentration of fungal pathogen contaminate environmental samples. The incubation period led to the appearance of an intense band of the PCR-product, these samples produced a positive detection even without incubation (Fig. S19a). For the incubated soil samples, the presence of this fungal pathogen was detected in all samples, thereby confirming that incubation led to the detection of the presence of *B. cinerea* inside the samples with a lower concentration of spores (100 spores g^{-1} of soil). Diguta *et al.* (2010) estimated the detection limit of *Botrytis cinerea* in grape berries to be 6.3 pg which corresponds to 540 spores. In the present study, the detection limit for spores inside soil and strawberry fruit without incubation was 100 spores g^{-1} of soil or fruit. In our study, *Colletotrichum* spp. were detected in a few samples. In the incubated strawberry fruit, which was naturally contaminated (Fig. 2), the presence of this fungus was detected inside all samples after 24 and 48 h of incubation. It was observed that the incubation period for the detection of *Colletotrichum* sp. was reasonable because without incubation the pathogen was not found (Fig. S13). By analysing the naturally contaminated environmental samples (Fig. 3), both of the extraction kits used led to the discovery of the presence of *Phytophthora* sp. in the same samples derived from naturally contaminated plants. Bonants *et al.* (1997) designed a species-specific primer for *P. fragariae* on the basis of the ITS region. In their work, a weak PCR-product was detected in the strawberry roots with low levels of contamination, but a much better PCR-product was found in the heavily infected roots. The other study on the detection of *P. fragariae* (Bonants *et al.*, 1997; Koprivica *et al.*, 2009; Schlenzing, 2009) was also based on 5.8S, ITS regions and nested PCR. In our study, the presence of *Phytophthora* sp. was detected using a single PCR analysis. The incubation of naturally contaminated strawberry did not lead to the improved detection of *Phytophthora* sp. (Fig. 2). When the soil and artificially contaminated strawberry samples were analysed, *Phytophthora* sp. was not detected in the soil (Figs 4, S15), but was only found in artificially contaminated strawberry, most particularly when the DNA was extracted using an FF kit (either incubated or not, Fig. S25c). Prigigallo *et al.*, (2015) detected the presence of *Phytophthora* sp. on soil and roots using a specific primer designed to be complementary to the ITS rDNA region. In comparing the two studies, the advantage of the method developed in this study is that it is based on a single conventional single PCR, not nested-PCR, while the assay proposed by Prigigallo *et al.* (2015) requires a semi nested-PCR. Finally, *Phytophthora* sp. was only detected in samples derived from infected plant tissues or strawberry fruit. These findings may be related to the structures of pathogens present in the soil, as sporangia are predominantly formed in the soil, whereas mycelium and zoospores do not remain viable in the soil for extended periods (Zan, 1962). A more effective way for *Phytophthora* sp. to survive within the soil is through oospore formation. The type of soil, the clayey and silty soils, may favour the survival of spores because water may leach them into the deeper soil layers (Zan, 1962). The soil pH may interfere with this process by inhibiting fungal activity because soil with a lower pH (< 5) presents a high degree of pathogen suppression on *Phytophthora* and in the study by Ann (1994) a quadratic relationship was found between soil pH and the sporangial germination of three *Phytophthora* sp. (*P. parasitica*, *P. palminova*, and *P. capsici*). These findings may explain the lack of *Phytophthora* sp. detection in soil samples, especially since the plantation was established on rather sandy acidic soils. The last fungal pathogen *Verticillium* sp. was detected inside both artificially and naturally contaminated samples. In the naturally contaminated samples, *Verticillium* sp. was only found in the shoots and roots of the strawberry plants, but not in the fruit (Fig. 3). By analysing the effect of incubation for the naturally contaminated strawberry samples, it was confirmed that incubation at 24°C had a positive influence on the detection of this fungus (Fig. 2). When eDNA was extracted from the soil using the ES kit, after 24 h of incubation, the presence of the tested pathogenic fungus was detected in the samples with the following spores concentrations (1 000-10 000 spores g^{-1} of soil, Fig. S15a), whereas after 48 h, the detection of *Verticillium* sp. was only observed in two samples with high spores concentrations (5 000-10 000 spores g^{-1} of soil, Fig. S14). By contrast, when the FF kit was used

to extract eDNA, the detection of the *Verticillium* sp. was only possible in artificially contaminated soil samples with high spore concentrations (5 000-10 000 spores g^{-1} of soil, Fig. S14). Kuchta *et al.* (2008) attempted to detect the presence of *V. dahliae* in both strawberry plants and soil through a single and nested-PCR test. They did not obtain a specific PCR-product from the plant and soil either through a single PCR test nor from a nested-PCR test using a primer design based on a region of the mitochondrial small rRNA subunit or ribosomal intergenic sequence. In comparing this work with our own study, a genera-specific primers designed based on functional genes were used, and the presence of *Verticillium* sp. was detected in artificially contaminated soil (detection limit 1 000 spores g^{-1}, Fig. S14) and strawberry (detection limit 100 spores g^{-1}, Fig. 4b). The lack of *Verticillium* sp. detection in the soil samples may be connected with the consideration that this fungus requires plant residues for mycelium development from the spores present in the soil and that the number of spores present in the soil was under the detection limit.

Analysing the results obtained from the field experiment, we can observe different trends depending on the resource analysed and the type of cultivar (Figs 5-6, S18). Regarding *Botrytis cinerea*, we cannot determine whether the bio-preparations we tested can prevent the infection caused by this pathogen, as the pathogen was not present in the control. Speaking instead of *Colletotrichum acutatum*, analysing the soil and leaf samples we were able to notice in the Honeoye and Vibrant cultivars, both in the irrigated and non-irrigated system, treatments P3 and P3+P1+P2 affected the abundance of the presence of *C. acutatum*. In contrast, for the Rumba cultivar we noticed that in the irrigated system the treatments P3+P2, P3+P1+P2, and P1+P2 had an effect while in the non-irrigated one they were P3+P2, P3+P1+P2, and P1+P2. Analysing the pathogenic oomycete fungus that different behaviours are revealed depending on the cultivars analysed. For the Honeoye cultivar in the non-irrigated system no treatment presented a reduction in the presence of this potential phytopathogen, while treatment P1+P2 presented a positive effect in reducing its presence. The same was observed for the Rumba cultivar, but only for the non-irrigated system did treatments P3 and P3+P1+P2 show positive results. For the last cultivar (Vibrant) we found for both systems a decrease in this potential phytopathogen after the application of treatment P3+P1+P2. As regards the previous pathogen, *Verticillium* sp., as regards soil and leaves, we did not detect any reduction in the abundance of the pathogenic fungus for the Vibrant and Rumba cultivars, while for Honeoye, they had a positive outcome in the system irrigated treatments P3+P1+P2 and P1+P2. Overall, we can observe that for *C. acutatum*, *Phytophthora* sp., and *Verticillium* sp., treatment P3+P1+P2 is the only one that is effective for all cultivars in both systems analysed (irrigated and non-irrigated).

This differentiation in the results produced by the strawberry fruit through the use of two different kits used for DNA extraction may be connected with the presence of an inhibitor, *e.g.* polysaccharides, phenol, polyphenols, salt (Kuchta *et al.*, 2008; Lilja *et al.*, 2006; Noh *et al.*, 2017; Pertile *et al.*, 2020; Schrader *et al.*, 2012; Wei *et al.*, 2008), which are co-extracted with eDNA. The concentration of these inhibition compounds changes according to the function of the life stage of the strawberry plants, thus it is very difficult to avoid their co-extraction with eDNA (Schlenzing, 2009). In our study, improved results of detection in plant materials were obtained after DNA extraction with the FF kit. The same phenomenon may be observed for the soil samples in which humic acids co-extracted with DNA are the main drawback of PCR-inhibition In the case of strawberry samples, we may also consider that the lack of the detection of these pathogens may be connected with the resistance of the strawberry variety (Lilja *et al.*, 2006), whereas for the soil, this may be connected with the presence of the *Trichoderma* sp. (Oszust *et al.*, 2020) or other antagonistic microorganisms inside the soil that can protect plants against fungal pathogens (Frąc *et al.*, 2018). Furthermore, it should be taken into consideration the fact that fungal and fungal-like pathogen DNA is only a small part of the eDNA extracted from the soil and strawberry (Kuchta *et al.*, 2008) and that the quantity of DNA from the pathogens change as a function of the age and stage of growth of the fungus (Atallah *et al.*, 2007).

5. CONCLUSIONS

The results demonstrated that developed assay using specific primers designed based on functional genes can be used as a molecular alternative for phytopathogens presence monitoring and routine investigation of environmental from strawberry plantations. The main innovation of this study may be to detect the presence of main potential phytopathogens from the genus *Botrytis*, *Colletotrichum*, *Phytophthora* and *Verticillium*, both in the soil and on plant material (roots, shoots, fruits), thereby facilitating the discovery of asymptomatic plants, as well as allowing for the quick and efficient management of diseases, this particularly applies to the detection of pathogens when they are inactive due to the formation of resistant structures, such as microsclerotia or oospores. This would allow farmers to act promptly by applying various procedures of infection control and plant protection. In addition, the developed assay may be used as a cost-effective method for the initial screening of newly established plantations.

Data Availability Statement: The datasets used and analysed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest: The authors declare that they have no conflict of interest.

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