

Apple Pomace Microbiome Carrying Fungal Load against Phytopathogens – Considerations Regarding Application in Agriculture and Horticulture

Karolina Oszust,* and Magdalena Frąć

This experiment evaluated the taxonomic diversity of the fungal community in conventional (AP) and organic (OAP) apple pomace using high-throughput sequencing, applying fungal genetic barcodes to functional guilds. The most abundant taxonomic groups identified in both AP and OAP were the genera *Aureobasidium*, *Cladosporium*, and *Alternaria*, classified into the pathotroph-saprotroph-symbiotroph guild. The phenotype microarray provided insight into the role of the apple pomace fungal community in the ecosystem. It is theorized that adding apple pomace to the soil may improve the bioavailability of bioresource-based polyols. Evaluation of the antagonistic ability of the AP fungal community and *Trichoderma atroviride* G79/11 strain against pathogenic fungi was performed. *Trichoderma* G79/11 developed well on apple pomace and revealed the antagonistic mode against tested fungal plant pathogens. Therefore, it could be applied to soil as a formulation of AP with spores or AP with *metaferm* biopreparation.

Keywords: Apple waste; Fungal diversity; Phytopathogens; Antagonism; *Trichoderma atroviride*; Agriculture

Contact information: Institute of Agrophysics, Polish Academy of Sciences, Doświadczalna 4, 20-290 Lublin, Poland; *Corresponding author: k.oszust@ipan.lublin.pl

INTRODUCTION

A wide array of scientific activities and their resulting products, including biofertilizers, microbial pesticides, and bio-control agents, used to fight plant pathogens are present at the interface of applied microbiology and horticulture (Ray and Ward 2008). The use of advanced microbiological techniques and tools to characterize the genetic and functional diversity of the microbial community has also been recently proposed as a strategy in biowaste eco-toxicological evaluation (Oszust and Frąć 2018; Oszust *et al.* 2018a).

Of large-scale horticultural waste, the apple juice industry generates a large amount. The waste takes the form of apple pomace, the by-product that results from apple processing and consists of apple skin, seeds, and stems (Wang *et al.* 2019). Approximately 75% of apple fruit is processed for juice or cider, and the remaining 25% of the weight of the fresh fruit constitutes biowaste. Apples (*Malus domestica*) are the favoured fruit of millions of people, and are widely grown in the temperate regions of the globe (Shalini and Gupta 2010). Poland is one of the major apple producers, with approximately 3170 Gg of apples produced every year (Lipiński *et al.* 2018).

Fruit pomace is a generally rich source of biological compounds and has become an important raw material from which to obtain various valuable by-products. Thus, it offers a logical basis for waste management and apple pomace is used for fuel and food

purposes, biotransformation, a source of fibre and pectin, microcrystalline cellulose (Shalini and Gupta 2010), nanocellulose (Szymańska-Chargot *et al.* 2019), and other bioactive compounds, such as organic acids and flavonoids (Mourtzinou and Goula 2019). Apple pomace application for agricultural purposes could be especially important for local apple processing companies surrounded by orchards and crops, which often lack sources of exogenous organic matter. The EU Thematic Strategy on Soil Protection lists the decline in organic matter as one of the main threats to soil quality, and calls for cultivation and agricultural production systems that will lead to an increase in its content (Soil Quality and Policy 2018).

The agricultural use of fruit pomace as a natural fertilizer promoting plant growth in organic farming has been studied previously (Mercy *et al.* 2014). Pomegranate, orange, sweet lime, and banana pomaces have promoted the growth of plants and have helped achieve higher yields, due to the organic matter and nutrients introduced. The risks and opportunities of organic farming have recently been summarized (Röös *et al.* 2018). It should be emphasized that additional fungal species may follow in the wake of biofertilizer application and may be unincorporated into the soil with almost any kind of biofertilizer (Frac *et al.* 2014).

Various microbial inhabitants may be described as indigenous representatives of biowaste. They may undertake a pivotal ecological role in influencing plant health as symbionts or decomposers when they are introduced to fields in the form of biofertilizers (Oszust *et al.* 2018a). In contrast, they may be pathogenic to plants, produce toxins, or cause mycoses (Presterl *et al.* 2018). To date, the mycological compositions of biofertilizers have been poorly analysed and described compared with analysis of their physicochemical properties (Oszust *et al.* 2018b).

However, biofertilizers may influence soil fungal biodiversity after their agricultural application. Therefore, the aims of the present study are as follows: [i] to assess the potential threats and benefits of introducing fungal representatives into the soil with apple pomace as a fertilizer, attributing fungal genetic barcodes to functional guilds, and thus evaluating the taxonomic diversity using high-throughput sequencing; and [ii] to provide insight into the role of fungal community maintenance in apple pomace ecosystem functionality. Herein, the specific respiration rate demonstrates a metabolic effect, in addition to biomass presented as a ratio development in the phenotype microarray. The methods of Pinzari *et al.* (2016) were followed and applied to the fungal community for the first time.

The statement that apple pomace carries a fungal load may also be considered from another point of view: namely, that waste is also a carrier of microbial beneficial strains, which are intended to be incorporated into the soil to fulfil a positive function. In that case, the indigenous microbial fungal species of the organic waste may be introduced into plant cultivation. Accordingly, it was discovered that fruit pomace, which is a solid waste, may be used as a growth substrate for microorganisms that inhibit plant pathogen development (Kalidas 1999).

The *Trichoderma* genus, a well-known fungus, is able to parasitize a great number of other soil-borne fungi that are pathogenic to plants. *Trichoderma* sp. comprises numerous biopreparations. Among others, *Trichoderma atroviride* G79/11 was previously described as a strain with cellulolytic potential (Oszust *et al.* 2017b), and based on the strain culture *metaferm*, a multi-enzymatic biopreparation was developed (Oszust *et al.* 2017a). However, no analysis has been performed to determine its antagonistic ability.

It was assumed that *Trichoderma atroviride* G79/11, which revealed a relatively high cellulolytic activity, would also develop well on apple pomace and demonstrate an antagonistic activity against pathogens. Therefore, the third aim of this study is to [iii] evaluate the associated antagonism between apple pomace and the G79/11 strain against four fungal pathogens that have been known to devastate soft fruit plantations.

These pathogens were *Colletotrichum* sp., *Botrytis* sp., *Verticillium* sp., and *Phytophthora* sp. *Colletotrichum* sp. is a burdensome fungal pathogen in modern agriculture. *C. acutatum* is one of the most harmful species of this genus; it causes anthracnose in plants such as strawberries and raspberries (Dolan *et al.* 2018; Forcelini *et al.* 2018). *Botrytis cinerea* is a causal agent of grey mould, and its resistance to fungicide renders it one of the most harmful pathogens affecting raspberry and strawberry plants as it diminishes otherwise effective management strategies (Kozhar and Peever 2019; Weber and Hahn 2019). The *Verticillium* genus includes two major species, *V. dahliae* and *V. alboatrum*, that cause losses in agriculture. *V. dahliae* causes wilt on economically important crops including strawberries and raspberries (Fan *et al.* 2018). It is also theorized that the *Phytophthora* species is responsible for most strawberry and raspberry losses in all production areas of the world (Nellist 2018). Scientists distinguish between two varieties of this species: *P. fragariae* var. *fragariae*, which can only infect strawberries, and *P. fragariae* var. *rubi*, which is native to raspberries. The co-occurrence of those pathogens may appear, *e.g.*, *Phytophthora rubi* and *Verticillium dahliae* in the form of late-summer disease symptoms in red raspberry fields (Weiland *et al.* 2017; Nellist *et al.* 2018). A universal natural enemy of those fungi is thus required in organic horticulture, especially because the worldwide area of organic cultivation has increased meaningfully over recent years (Kiełbasa 2015).

EXPERIMENTAL

Materials

Apple pomace

Two types of apple waste were used for the experiments: apple pomace (AP), a waste consisting of a mixture of red-coloured apple varieties grown conventionally in the Grójec area of Poland, obtained from a local apple juice-processing factory; and organic apple pomace (OAP) of the Gala variety that was ecologically grown in Trentino-Alto Adige, Italy. The content of mineral ingredients (NPK) and the organic matter content were evaluated. The phosphorus (P) level was calorimetrically determined and the potassium (K) content was estimated by flame photometry according to the Spurway method (Spurway and Lawton 1949). The total nitrogen and organic matter contents were assessed according to the Kjeldahl and weight method, respectively. All analyses were determined at the District Chemical and Agricultural Station in Rzeszów, Poland. The results were obtained in the form of mean values. All chemical analyses of the tested waste were performed in triplicate.

Methods

Next generation sequencing - Meta-barcoding

An analysis of the fungal community structure of apple pomace was performed on the basis of the region of Internal Transcribed Spacers 1 (ITS1). The set of the following primers ITS1FI2: 5'-GAACCGCGGARGGATCA-3' (Schmidt *et al.* 2013) and 5.8S:

5'-CGCTGCGTTCTTCATCG-3' (Vilgalys 1992) were used to amplify the selected region. The polymerase chain reaction (PCR) was completed in a Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA, USA) according to the conditions included in the manufacturer's protocols. The DNA library was sequenced using an MiSeq platform (Illumina Inc., San Diego, CA, USA) with pair-end (PE) technology, 2 x 250 bp using the v2 Illumina kit following the manufacturers' instructions (Genomed S.A., Warsaw, Poland). MiSeq Reporter (MSR) v2.6. software (Illumina Inc., San Diego, CA, USA) was used for a preliminary elaboration of the data and the Quantitative Insights into Microbial Ecology (QIIME) tool (Illumina Inc., San Diego, CA, USA) was used to process the raw sequencing data (Caporaso *et al.* 2010). The analysis included the following steps: reading quality evaluation, removing low quality sequences and chimeras, and generating operational taxonomic units (OTUs) that were defined by clustering at 97% similarity. The taxonomical classification of the OTUs was achieved using a Basic Local Alignment Search (BLAST) against the UNITE database.

A bioinformatics assay was based on the reference sequence database, Greengenes_13_05 (DeSantis, Jr. *et al.* 2006b), and was performed using an algorithm from Qiime software (Caporaso *et al.* 2010). The analysis included the following steps: demultiplexing of samples and adaptor cutting, conducting quality analysis, determining taxonomic composition, and performing diversity analysis. Sequences that were over 97% identical were grouped into one OTU using a distance-based OTU program. The application of MiSeq Reporter v2.3 (Illumina Inc., San Diego, CA, USA) allowed for species-level classifications. The taxonomy database for the metagenomics workflow was the Illumina version of the Greengenes database (DeSantis, Jr. *et al.* 2006a). FUNGuild was used to taxonomically parse fungal OTUs using the ecological guild (Nguyen *et al.* 2016).

Phenotype microarray

Phenotype fingerprinting of apple pomace (AP and OAP) fungal community was determined using the Biolog® System FF MicroPlates (Biolog®, Hayward, CA, USA), expressed as the ratio between the values of substrate use (respiration, OD 490 nm) and the growth pattern (biomass production, OD 750 nm); seven carbon source groups were evaluated (Pinzari *et al.* 2016, 2017). The protocol followed that of Jeszka-Skowron *et al.* (2018). The analyses were performed in three technical replications (n=3). Results are presented as mean values, and standard errors are provided.

Microbial Strains and *metaferm* Biopreparation

The following fungal pathogens were used in the study: two strains of *Colletotrichum* sp. (G166/18 and G168/18) isolated from infected strawberry fruit, two strains of *Botrytis* sp. (G277/18 and G276/18), two strains of *Verticillium* sp. (G296/18 and G297/18) isolated from infected strawberry roots, and two strains of *Phytophthora* sp. (G373/18 and G368/18) from the collection of the Research Institute of Horticulture in Skierniewice (Skierniewice, Poland). Concerning the beneficial and possibly antagonistic strain of *Trichoderma atroviride* G79/11, its origin and characteristics were described by Oszust *et al.* (2017b). *Metaferm* is a multi-enzymatic biopreparation previously developed and described by Oszust *et al.* (2017a). It reveals hydrolytic activities such as those involving: xylanase, β -glucosidase, carboxymethyl cellulase, poligalactouronase, pectinesterase, amylase, lactase, and protease (Frąc *et al.* 2014; Oszust *et al.* 2017a). The *metaferm* used for the purposes of the experiment was stored at -20 °C for 4 years.

Antagonism Experiment

To evaluate the interactions between the antagonistic variants and the pathogens in the *in vitro* culture, the following antagonistic variants were used: (1) *Trichoderma atroviride* (G79/11) (5-mm fragments of potato dextrose agar (PDA) cultured for 10 days), (2) AP (0.2 g), (3) AP inoculated with *Trichoderma atroviride* (AP-G79/11) (0.2 g AP inoculated *via* an inoculation needle with spores of G79/11), (4) *metaferm* (MET) (100 μ L into the 5-mm diameter hole in PDA), and (5) AP with *metaferm* (AP-MET) (0.2 g AP and 100 μ L MET). They were tested against four selected pathogenic species (*Colletotrichum* sp., *Botrytis* sp., *Verticillium* sp., and *Phytophthora* sp.) represented by two isolates each.

The discs of 5-mm diameter PDA (with antibiotics addition) containing 10-day-old cultures of pathogenic fungi were placed on a Petri dish with 20 cm³ of PDA. The scheme of the antagonism experiment is shown in Fig. 1. Controls of all of the pathogenic strains without any treatment were provided. After incubating the pathogenic fungus and antagonism variant at 22 °C for approximately 5 days, the horizontal culture and vertical growth diameter (mm) were measured. The experiment was set up in triplicates (n=3). Results were presented as mean values with standard error provided.

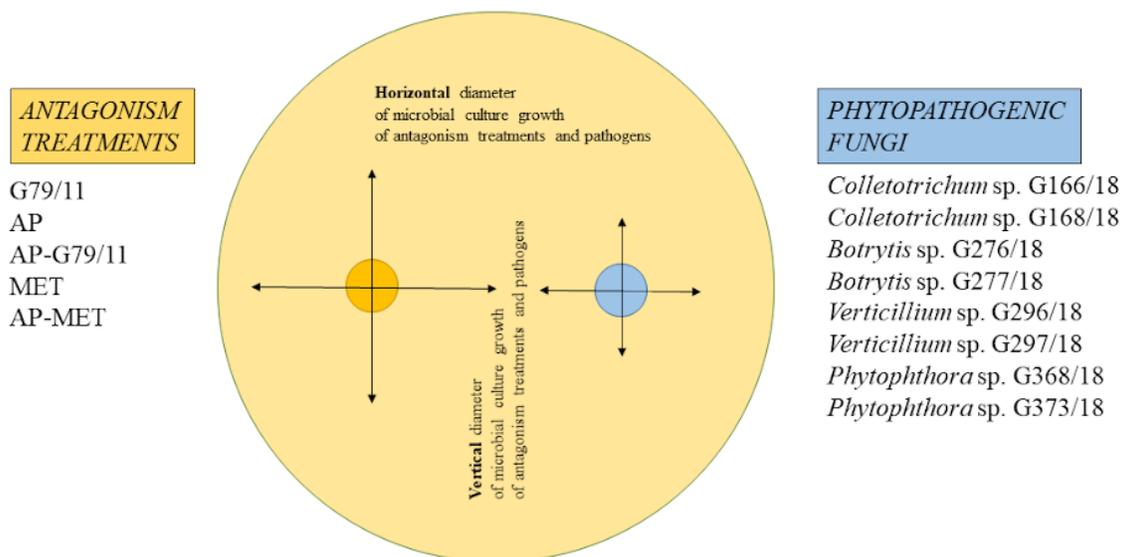


Fig. 1. Antagonism experiment diagram (explanations: G79/11 - *Trichoderma atroviride* G79/11; AP – apple pomace; AP-G79/11 – apple pomace inoculated with *Trichoderma atroviride* G79/11; MET – *metaferm* multi-enzymatic biopreparation based on post-culture, concentrated liquid of *Trichoderma atroviride* G79/11 in soy flour-cellulose-lactose medium; AP-MET – apple pomace with the addition of *metaferm*; n = 3)

RESULTS AND DISCUSSION

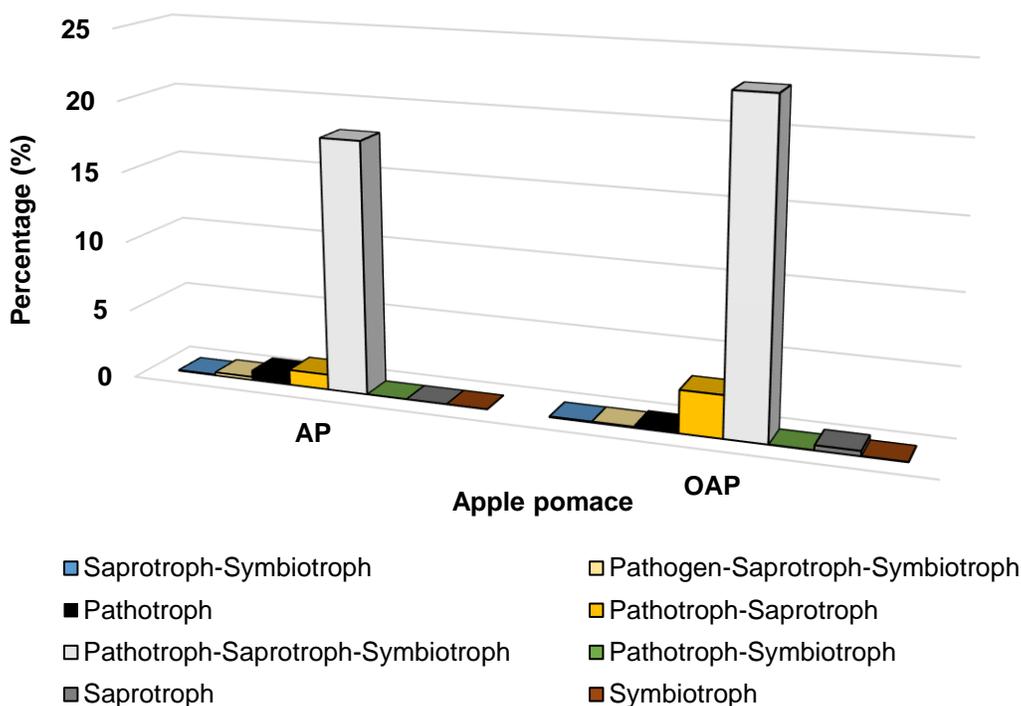
Apple pomace and OAP exhibited differences with regards to their chemical properties, as shown in Table 1. The latter contained twice as much total nitrogen and phosphorus (1.18% and 0.14%, respectively) as AP. Organic matter and potassium content were relatively equal in AP and OAP (0.8% and 98%, respectively).

Table 1. Chemical Properties of Apple Pomace

Apple Pomace	Dry Matter	Total Nitrogen (ON)	Phosphorus (P)	Potassium (K)	Organic Matter (OM)
	%				
AP*	26	0.496	0.085	0.81	98.04
OAP**	23	1.184	0.140	0.70	97.93

* Apple pomace; ** organic apple pomace; n = 3

The study evaluated the share of fungal OTUs in apple pomace organized into groups that referred to trophic modes (Fig. 2). Among all of the obtained OTUs for AP, 20% were assigned and organized into trophic modes. For OAP, the total assignment was 26%. Therefore, 74% to 80% of the OTUs found were unassigned. In detail, the most numerous group of AP (constituting 18% of all OTUs) and of OAP (constituting 23% of all OTUs) was assigned to the pathotroph-saprotroph-symbiotroph group. The pathotroph-saprotroph group was the second organized entry detected, but much less numerous (1% in AP and 3% in OAP).

**Fig. 2.** The share of fungal OTUs entries organized into groups that referred to trophic modes in apple pomace

The relative abundance (%) of fungal OTUs ID entries have been qualified directly into taxa and indicated with trophic modes and guilds, as shown in Table 2. Both yeasts and moulds were found, including soft rot, endophyte pathogens, fungal parasites, likely

opportunistic human pathogens, biotrophic, necrotrophic, or saprobic, on various plant tissues or/and necrotrophic on stems or leaves.

The most abundant taxonomic groups at the genus level that were identified in both AP and OAP were *Aureobasidium* sp. (2.75% and 12.7%, respectively), *Cladosporium* sp. (4.16% and 7.31%, respectively), and *Alternaria* sp. (0.93% in AP and 2.56% in OAP) were classified into pathotroph-saprotroph-symbiotroph group. *Malassezia* sp. representatives (0.1% in AP and 0.05% in OAP) and *Leptosphaeria* sp. were also found in both AP and OAP, though in a smaller amount (0.5% and 0.07%, respectively). The following genera: *Phoma*, *Acremonium*, *Mycosphaerellaceae*, and *Exobasidium* primarily occurred in AP. The genera *Leptosphaeriaceae*, *Didymella*, *Sporobolomyces*, and *Rhodotorula* were only found in OAP.

Among all the taxa described, only *Malassezia* sp. (< 0.1% in AP and OAP) and *Sporobolomyces* sp. (0.2% in OAP) were not thought to behave as plant pathogens. The animal pathogens in both AP and OAP were *Cladosporium* sp., *Alternaria* sp., and *Malassezia* sp.; in AP, *Acremonium* sp.; and, in OAP, *Didymella* sp. and *Rhodotorula* sp. Most of entries were classified as probable saprotrophs. Only two representatives were assigned to perform fungal parasitism, *Acremonium* sp. (0.15% AP) and *Sporobolomyces* sp. (0.2% OAP).

An evaluation of *in vitro* antagonism treatments on PDA against *Colletotrichum* sp. isolates is shown with photographic documentation in Fig. 3 (a to d), *Botrytis* sp. in Fig. 4 (a to d), *Verticillium* sp. in Fig. 5 (a to d), and *Phytophthora* sp. in Fig. 6 (a to d). *Botrytis* sp. and *Colletotrichum* sp. were the most expansive in this experiment. *Botrytis* sp. isolates outgrew the petri plate (diameter 80 mm), which meant that it was the most expansive sample among the pathogens tested. *Colletotrichum* sp. growth was slightly lower (60 mm). The slightest growth was noted for the *Verticillium* sp. (< 40 mm) and *Phytophthora* sp. isolates (< 12 mm).

Almost all proposed antagonism treatments were effective for pathogenic fungi growth inhibition, which was demonstrated by the decrease in horizontal and vertical growth diameter. *Colletotrichum* sp. isolates had an approximately 70% diameter decrease in G79/11 and AP-G79/11 strains, and 30% for AP, MET, and AP-MET. For *Botrytis* sp. G279/18, a 50% decrease was noted for AP, G79/11, and AP-G79/11, and 25% for MET and AP-MET. For *Botrytis* sp. G277/18, a 50% decrease was observed for G79/11, AP-G79/11, AP-MET, and a 25 to 30% decrease for AP and MET. In contrast, for *Verticillium* sp. G269/18 and G297/18, a 50% decrease for AP, and 70% decrease was noted for the rest of tested treatments. The only exception was *Phytophthora* sp. G373/18, which was not influenced by antagonists. The isolate *Phytophthora* sp. G368/18 was revealed to be immune.

The inhibition effect differed with respect to the particular treatment. Generally, AP treatment revealed the lowest inhibition activity. However, the direct addition of *Trichoderma atroviride* G79/11 to AP in the form of spores (AP-G79/11), or indirectly with *metaferm* (AP-MET), predominantly improved the efficacy of its antagonistic activity. This improvement was manifested by the overgrowth of G79/11. For *Colletotrichum* sp. G166/18 (Fig. 3c) and *Verticillium* sp. G269/18 (Fig. 5c), no G79/11 development was noted in the AP-MET treatment, and other fungi exceeded its growth. As shown in various photos (Figs. 3c, 3d, 4c, 4d, 5c, 5d, 6c, and 6d), for AP treatment, macroscopic evaluation showed that AP treatment favoured development of different species, demonstrating a probable antagonistic treatment.

Table 2. Relative Abundance (%) of Fungal OTUs with Trophic Modes and Guilds Identified

OTU ID	Trophic Mode	AP	OAP	Taxon	Guild	Growth Morphology	Notes	Citation/Source
		%						
New.Reference OTU17	Pathotroph-Saprotroph-Symbiotroph	8.74	0.06	Phoma	Endophyte-Dung Saprotroph-Lichen Parasite-Litter Saprotroph-Plant Pathogen-Soil Saprotroph-Wood Saprotroph	Microfungus	Soft rot	Lagarde <i>et al.</i> (2018); Kim <i>et al.</i> (2019)
SH216250.07FU_EF679363_refs		4.16	7.31	Cladosporium	Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Wood Saprotroph	Microfungus	-	Chen <i>et al.</i> (2018); Lagarde <i>et al.</i> (2018)
New.Reference OTU140		2.75	12.7	Aureobasidium	Endophyte-Epiphyte-Plant Pathogen-Undefined Saprotroph	Facultative Yeast	-	Rathnayake <i>et al.</i> (2018)
New.CleanUp.ReferenceOTU 2031		1.00	0.00	Phoma	Endophyte-Dung Saprotroph-Lichen Parasite-Litter Saprotroph-Plant Pathogen-Soil Saprotroph-Wood Saprotroph	Microfungus	Soft rot	Lagarde <i>et al.</i> (2018); Kim <i>et al.</i> (2019)
New.CleanUp.ReferenceOTU 4820		0.93	2.56	Alternaria	Animal Pathogen-Endophyte-Plant Pathogen-Wood Saprotroph	Microfungus	Soft rot	Lincoln <i>et al.</i> (2018)
New.CleanUp.ReferenceOTU 11423		0.15	0.00	Acremonium	Animal Pathogen-Endophyte-Fungal Parasite-Plant Pathogen-Wood Saprotroph	Microfungus	Soft rot, endophyte pathogen, likely opportunistic human pathogen	Selvakumar and Panneerselvam (2018)
New.CleanUp.ReferenceOTU 8326	Pathotroph-Saprotroph	0.77	0.00	Mycosphaerellaceae	Plant Pathogen-Undefined Saprotroph	Microfungus	Biotrophic, necrotrophic or saprobic on various plant tissues	Santini <i>et al.</i> (2018)
New.CleanUp.ReferenceOTU 5116		0.10	0.05	Malassezia	Animal Pathogen-Undefined Saprotroph	Facultative Yeast	Skin diseases	Theelen <i>et al.</i> (2018)

New.CleanUp. ReferenceOTU 900		0.02	0.42	Leptosphaeriaceae	Plant Pathogen-Wood Saprotroph	Microfungus	Saprobic or weakly necrotrophic on stems or leaves	Chen <i>et al.</i> (2018)
New.CleanUp. ReferenceOTU 14202		0.02	0.46	Didymella	Animal Pathogen-Plant Pathogen-Undefined Saprotroph	Microfungus	-	Salehi <i>et al.</i> (2019)
New.CleanUp. ReferenceOTU 609		0.00	0.20	Sporobolomyces	Fungal Parasite-Litter Saprotroph	Yeast	-	Nagai (2018)
New.CleanUp. ReferenceOTU 5551	Pathotroph	0.50	0.07	Leptosphaeria	Plant Pathogen	Probable	-	Fudal <i>et al.</i> (2018)
New.CleanUp. ReferenceOTU 11223		0.19	0.00	Exobasidium	Plant Pathogen	-	-	Karakaya and Dikilitas (2018)
SH194775.07F U_AF444292_r efs		0.02	1.82	Rhodotorula	Animal Endosymbiont-Animal Pathogen-Endophyte-Plant Pathogen-Undefined Saprotroph	Yeast	Opportunistic human pathogen	Sen <i>et al.</i> (2019)

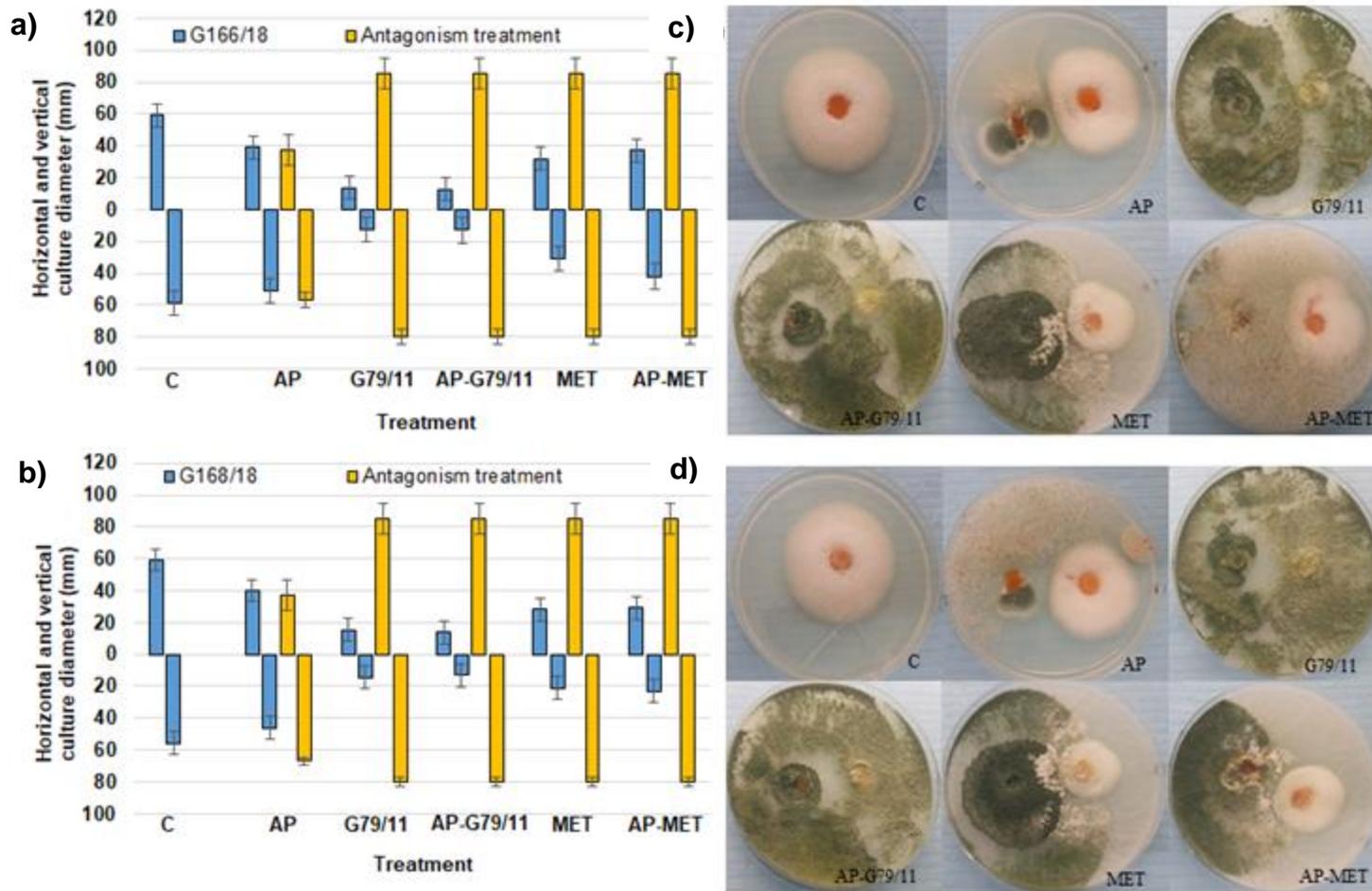


Fig. 3. Evaluation of *in vitro* antagonism treatments on the plate with potato dextrose lab (PDA) agar against *Colletotrichum* sp. (a) strain G166/18 and (b) strain G168/18, with photographic documentation (c) and (d), respectively. Explanations: C is pathogen growth control; antagonism treatments are as follows: AP, apple pomace; G79/11, *Trichoderma atroviride* G79/11; AP-G79/11, apple pomace inoculated with *Trichoderma atroviride* G79/11; MET, *metaferm* biopreparation; AP-MET, apple pomace with addition of *metaferm*; n = 3, the error bars represent standard error.

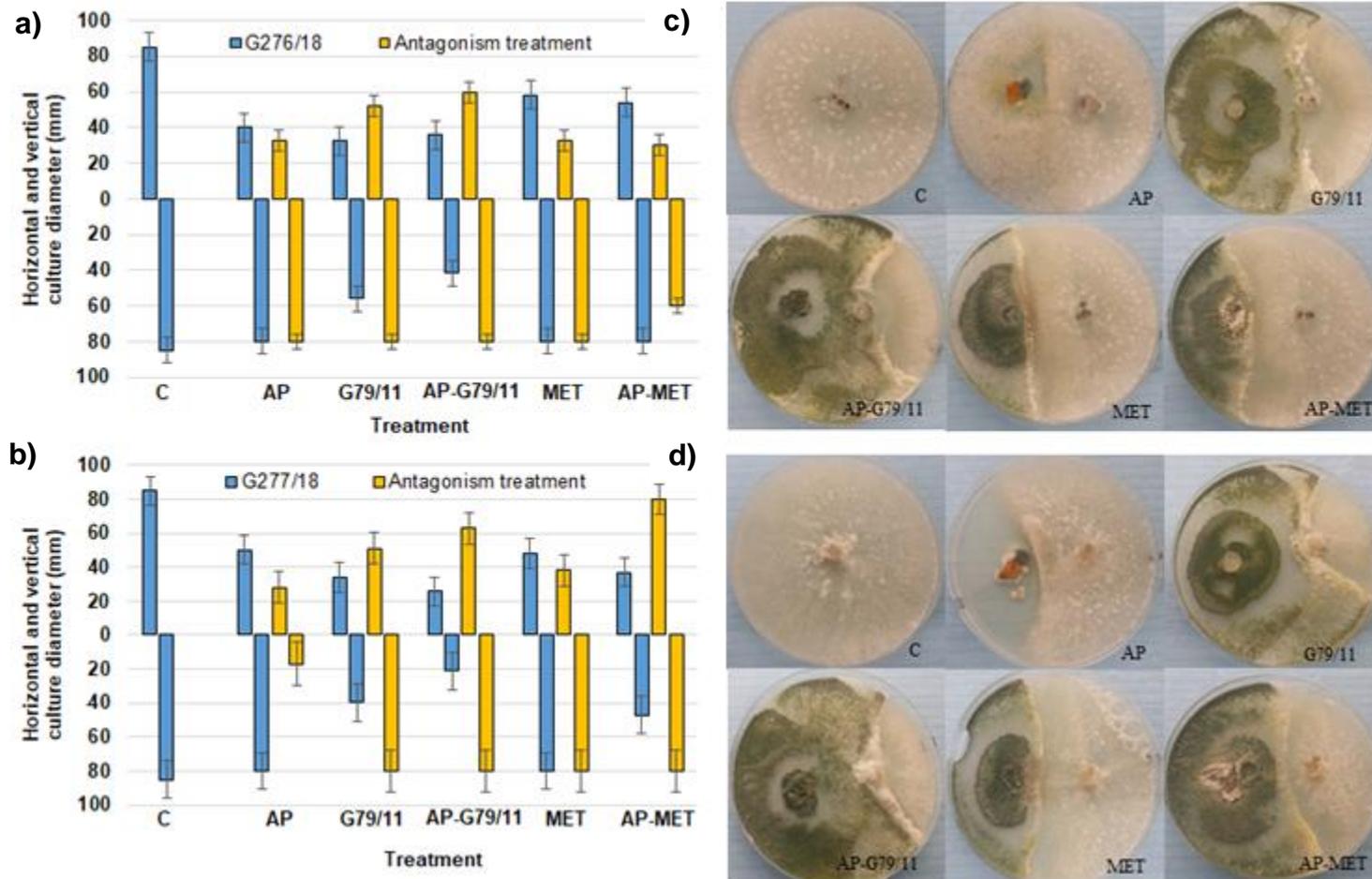


Fig. 4. Evaluation of *in vitro* antagonism treatments on the plate with PDA against *Botrytis* sp. (a) strain G276/18 and (b) strain G277/18, with photographic documentation (c) and (d), respectively. For explanations please see Fig. 3 caption.

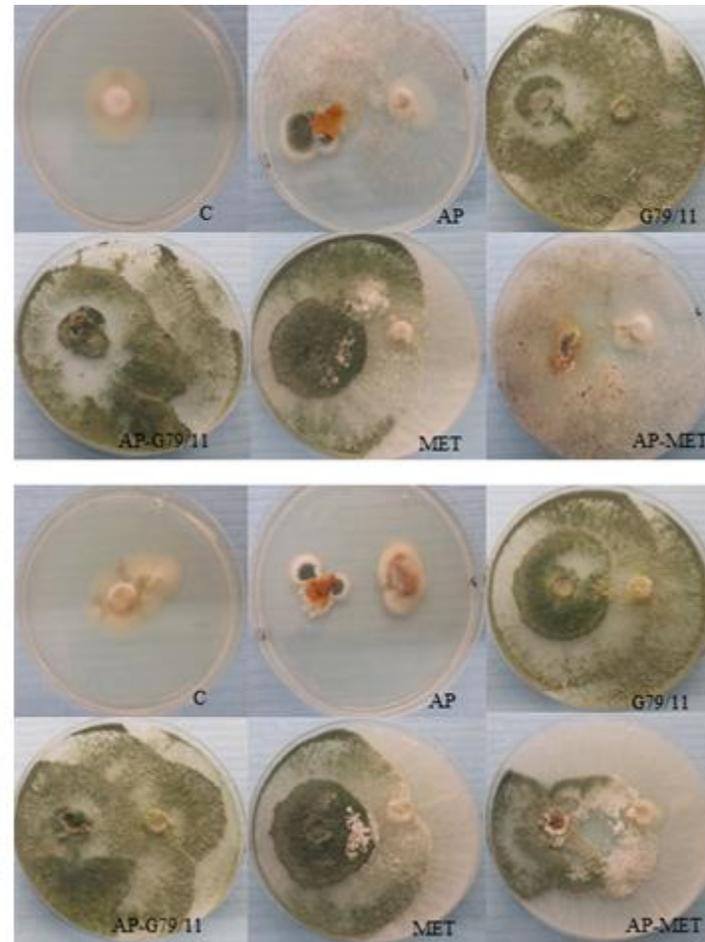
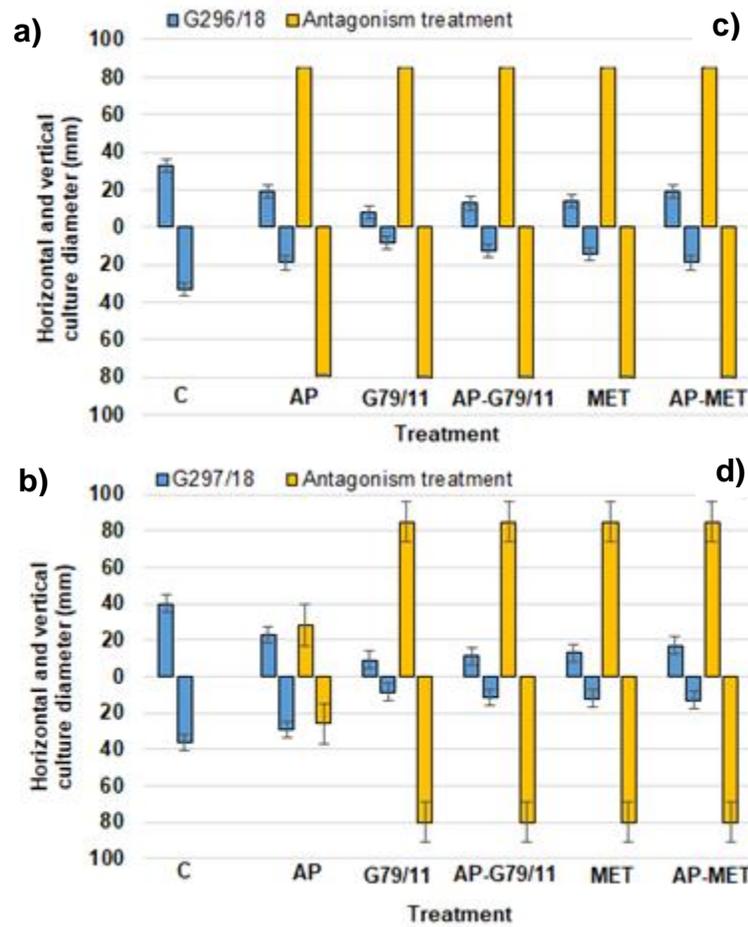


Fig. 5. Evaluation of *in vitro* antagonism treatments on the plate with PDA against *Verticillium* sp. (a) strain G296/18 and (b) strain G297/18, with photographic documentation (c) and (d), respectively. For explanations please see Fig. 3 caption.

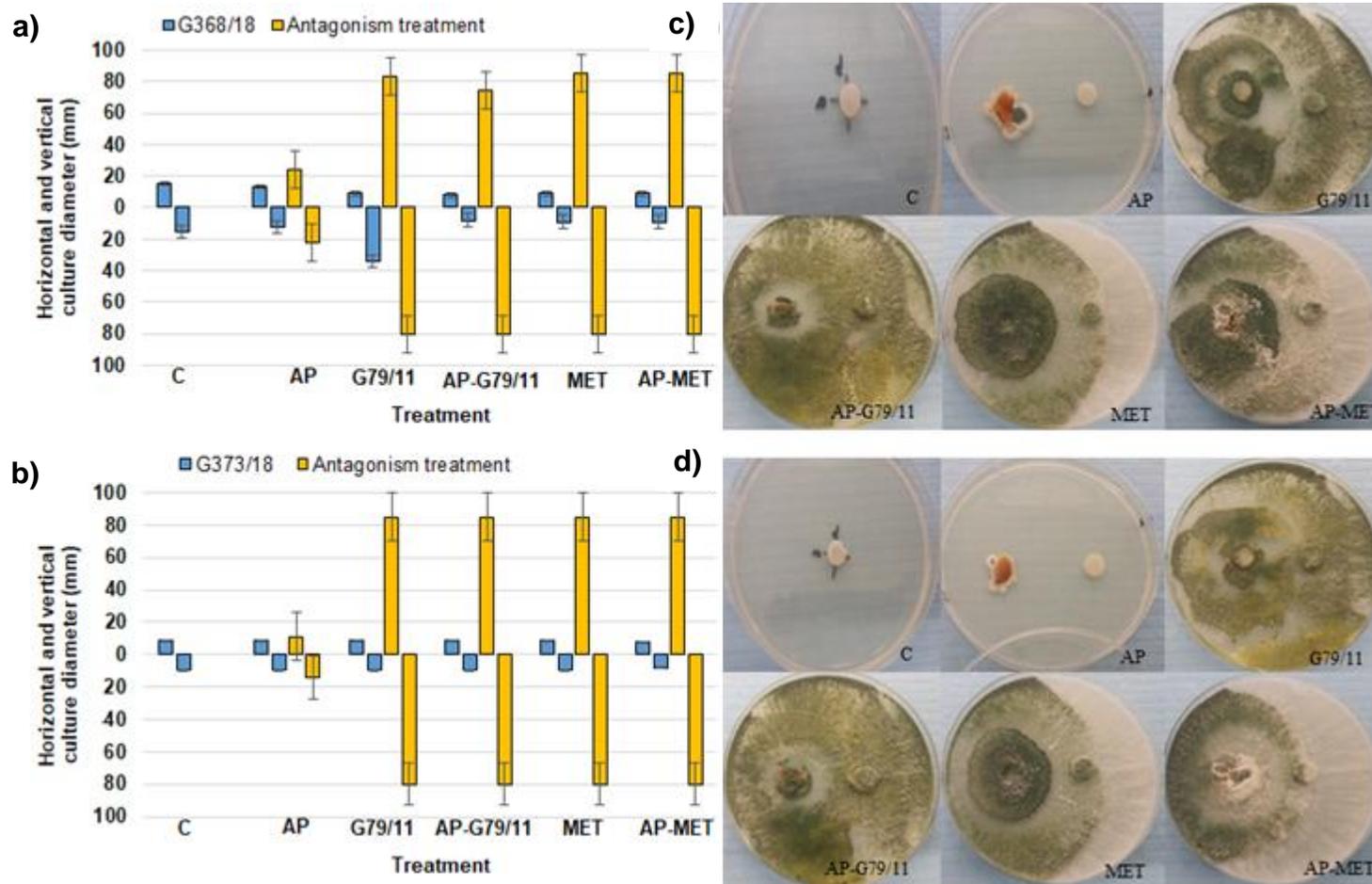


Fig. 6. Evaluation of *in vitro* antagonism treatments on the plate with PDA against *Phytophthora* sp. (a) strain G368/18 and (b) strain G373/18, with photographic documentation (c) and (d), respectively. For explanations, please see Fig. 3 caption.

Figures 7a and 7b show the absorbance normalized values and the ratio between the values of substrate use (respiration) and growth patterns (biomass production) by the fungal community, for particular groups of substrates on the FF plate, for AP and OAP, respectively. For AP (Fig. 7a), the ratio values were varied, ranging from 0.9 in the polyols group to 1.86 in aliphatic organic acids. Polyols generated the largest functional response (both in terms of growth and substrate use). More modest responses were generated by the following substances (in descending order): oligosaccharides, glucosides, hexoses, TCA-cycle intermediates, L-amino acids, peptides, pentoses, biogenic and heterocyclic amines, hexosamines, and heptose. Other groups, such as polysaccharides, sugar acids, and aliphatic groups, produced the smallest functional response. In OAP (Fig. 7b), the ratio values were stable and ranged from 1.0 to 1.8 for most of the substrate groups, and to 6.4 for pentoses. The functional responses were as follows (from the highest to the lowest): polyols, heptoses, oligosaccharides, polysaccharides, TCA-cycle intermediates, biogenic and heterocyclic amines, glucosides, others group, aliphatic organic acids, L-amino acids, sugar acids, hexosamines, pentoses, hexoses, and peptides.

Apple Pomace Taxonomic Composition and Function

The differences in the taxonomic compositions of fungal communities in apple pomace from conventionally grown apples and organic apple pomace were revealed. Abdelfattah *et al.* (2016) also found different population patterns and structures between organic and conventional apples. The findings of this study were in agreement with these results. Several unique taxa were exclusively detected in organic apples, suggesting that agricultural management practices may have been a contributing factor in determining the taxa present in the plant material.

Previous studies indicated that apple surfaces teem with a wide variety of microorganisms, mainly fungi that are closely associated with postharvest deterioration in the fruit (Shen *et al.* 2018). In particular, such studies reported an increased level of *Penicillium sp.*, *Aspergillus sp.*, *Mucor sp.*, and *Botrytis sp.* during apple storage; however, in the present study, none of the representatives of this genus were found in apple waste. Additionally, this study found the presence of *Acremonium sp.* representatives in apple pomace and organic apple pomace, but Shen *et al.* (2018) suggested that their proliferation is uncommon in the fungal communities of apple fruits during storage. It was previously stated that fruit surfaces also contain numerous unidentified fungi (Graça *et al.* 2015). The same results were demonstrated in this research. However, the present results also importantly contribute new knowledge concerning the fungal community composition in apple pomace, namely, the high-throughput sequencing technology approach.

Nonetheless, the ecological roles of the already identified barcode entries play a negative role as both a potential plant or animal pathogen, and/or a positive role as a saprotroph or fungal parasite, or/and endophyte/epiphyte. Therefore, its agricultural application may reveal some positive aspects by supplying the soil with an additional, reasonable amount of exogenous organic matter, as well as with nitrogen, phosphorus, and potassium. Apple pomace also carries a great number of fungal saprotrophs, and thereby ensures the circulation of organic matter. In contrast, the prospect of applying apple pomace in agricultural purposes may also pose disadvantages because many of its fungal representatives are phytopathogens. Previously, this problem was identified by Oszust *et al.* (2018b), who found the fungal pathogen *Petriella setifera* in industrial compost that consisted of sewage sludge from wastewater treatment, sawdust and biodegradable waste from gardens and from parks, soil, medicinal plants extracts, and lime sludge.

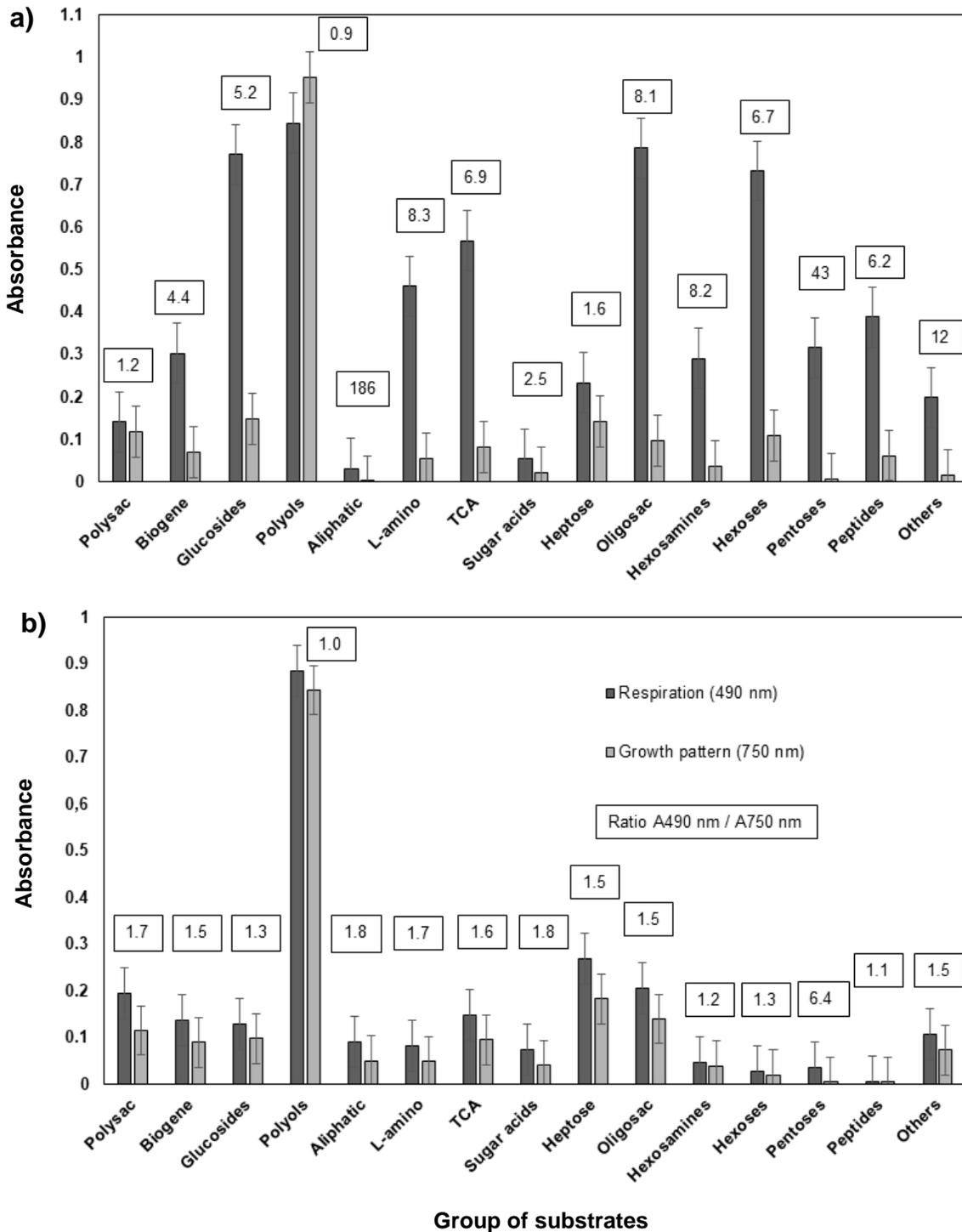


Fig. 7. The absorbance normalized values of substrates use and growth pattern fungal community of a) AP and b) OAP based on FF Biolog® Plate. The ratio between values of substrate use (respiration, OD 490 nm) and growth pattern (biomass/turbidity production, OD 750 nm) for each particular group of substrates is shown in frames. Explanations: Polysac, polysaccharides; Biogene, biogene, and heterocyclic amines; Aliphatic, aliphatic organic acids; L-amino, L-amino acids; Oligosac, oligosaccharides; n = 3; the error bars represent standard error.

In many orchards, the shredded branches resulting from pruning trees or raspberry bushes are left in inter-row spaces and under trees. This waste wood containing lignin is persistent in nature. Apple pomace may be used to stimulate the growth of white-rot fungi in soil (Cea *et al.* 2010), which is another benefit of apple pomace field application. However, because fresh apple pomace is an acidic substrate with a considerable buffering capacity—attributable to malic acid and its salts (Hang and Walter 1989)—the appropriate dose of apple pomace should be calculated and adjusted to the pH value of the target soil.

In the research of Pinzari *et al.* (2016) and Pinzari *et al.* (2017), when comparing the ratios obtained for the two species of fungi (pure isolates), for example, *Stachybotrys chartarum* and *Minimedusa polyspora*, evidence was found that the two organisms have differing behaviour in the presence of different groups of substrates. The same was revealed in the present experiments concerning fungal communities of apple pomace. This finding is probably related to the manner of apples production (conventional or organic farming) and the apple variety composition, which regulates its fungal community functionality. Additionally, Arrigoni *et al.* (2018) suggests that tissue age and plant genotype affect the microbiota of apple and pear bark.

The greatest diversity of ratio values for a particular group of substrates in apple pomace compared with organic apple pomace (Fig. 3) suggests that the apple pomace fungal community has the ability to respond to a larger variety of substrates. However, as Pinzari *et al.* (2017) theoretically describes, a lower ratio results in a more efficient metabolism. That criterion was met for organic apple pomace. A low ratio, compared with a high one, indicates that a relatively large fungal biomass developed without consuming too much substrate. The opposite situation occurred with regard to apple pomace: a small biomass (low OD values at 750 nm) yielded high respiration rates (high OD values at 490 nm), indicating a stressful metabolic situation.

According to this interpretation, which, in some cases, may be clearly and definitely applied, fungi in the apple pomace obtained by conventional farming methods appeared to be more compatible with polyols and polysaccharides. The fungi in the organic apple pomace showed high efficiency when growing on most of the tested substrates, and especially on polyols (excluding pentoses). That apple pomace from conventional and organic farming carries a fungal load with high tendency to grow on substrates, such as polyols, is useful when considering its potential agricultural and horticultural applications. It is theorized that adding apple pomace to soil may improve the processing of bioresource-based polyols, including the lignin or soy oil-derived polyols discussed above (Luo *et al.* 2018).

***Trichoderma atroviride* G79/11 Formulation on Apple Pomace**

Trichoderma atroviride G79/11 demonstrated cellulolytic activity (Oszust *et al.* 2017b). Moreover, the study indicated that this fungus also developed well on apple pomace and assumed an antagonistic mode against *Colletotrichum* sp., *Botrytis* sp., *Verticillium* sp., and *Phytophthora* sp. isolates. Therefore, apple pomace with *T. atroviride* G79/11 formulation could be applied in the field as a vehicle to suppress plant pathogens. Many other scientists have also examined the production of antimicrobial compounds by studying the *Trichoderma* sp., and have discovered that these fungi have the ability to produce toxic metabolites used by the fungi to inhibit the growth of other competitors in the ecological niche (pathogenic fungi) (Berg *et al.* 2004). Due to their ability to produce chitinase, many representatives of fungi belonging to the *Trichoderma* sp. are able to degrade pathogen cell walls (Larkin 2016).

The formulations proposed in this paper do not require the addition of nitrogen, or the adjustment of the pH, as Kalidas (1999) describes is necessary for the growth of antagonistic microorganisms in solid waste such as apple, strawberry, and/or cranberry pomace. Apple pomace, with the presented chemical composition, is primarily indicative of the content of cellulose (*ca* 8.8 g/100 g d.m.) (Szymańska-Chargot *et al.* 2019), which is a suitable substrate by which to develop a strain with the high cellulolytic activity of *Trichoderma atroviride* G79/11.

The tested multi-enzymatic biopreparation *metaferm*, based on the concentrated submerged culture in soy flour-cellulose-lactose medium of *Trichoderma atroviride* G79/11, produces conidial spores that are virile enough to initiate growth on apple pomace. It seems that long-term *metaferm* storage does not negatively influence *Trichoderma atroviride* G79/11 spores in their ability to thrive and demonstrate antagonistic activity. It is also possible that the inherent pathogens of apple pomace, described above, may be suppressed by the spread and prospering of *Trichoderma atroviride* G79/11.

CONCLUSIONS

1. The most abundant taxonomic genus identified in both AP and OAP were *Aureobasidium*, *Cladosporium*, and *Alternaria*, classified into the pathotroph-saprotroph-symbiotroph groups.
2. Unique taxa were exclusively detected in organic apples, suggesting that agricultural management practices may have been a contributing factor in determining the taxa present. *Phoma*, *Acremonium*, *Mycosphaerellaceae*, and *Exobasidium* were mainly found in AP. *Leptosphaeriaceae*, *Didymella*, *Sporobolomyces*, and *Rhodotorula* were found only in OAP.
3. The ecological roles identified for fungi are complex, as they may play a negative role as plant or animal pathogens, and they may simultaneously play a positive role as a saprotroph or fungal parasite, endophyte or epiphyte.
4. The indigenous fungal representatives of apple pomace may be introduced into the soil ecosystem, and they may undertake ecological roles influencing plant health as symbionts, decomposers, or pathogens.
5. The *Trichoderma atroviride* G79/11 strain has an antagonistic potential against *Colletotrichum* sp., *Botrytis* sp., *Verticillium* sp., and *Phytophthora* sp., and develops well on apple pomace. Therefore, *Trichoderma atroviride* G79/11 may be applied to the soil. It has been proven that both formulations of AP with G79/11 spores or AP with *metaferm* biopreparation act effectively against fungal pathogen.

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Karolina Oszust designed the study, collected the data, interpreted the results, and wrote the manuscript draft. Magdalena Frać provided ideas, designed the study,

participated in the interpretation of results, performed reviews, and made corrections of the manuscript. Both authors read and approved the final manuscript.

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