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Low density polyethylene degradation by filamentous fungi☆

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ABSTRACT

Polyethylene (PE) is the most abundant non-degradable plastic waste, posing a constant and serious threat to the whole ecosystem. In the present study, the fungal community of plastic wastes contaminating a landfill soil has been studied. After 6 months of enrichment, 95 fungi were isolated, mostly belonging to the Ascomycota phylum. They were screened under *in vitro* condition: most of fungi (97%) were capable of growing in the presence of PE powder (5–10 g L^{-1}) as sole carbon source. *Fusarium* strains better tolerated high concentration of PE. Up to 13 strains were chosen for further degradation trails, where the process was monitored by respirometry tests and by observing changes in PE chemical and physical structure by FTIR analysis and SEM images. Major results were observed for *Fusarium oxysporum*, *Fusarium falciforme* and *Purpureocillum lilacinum*, as they caused strong oxidation phenomena and changes in the PE film morphology. Results suggested that the initial oxidation mechanisms targeted first the methyl terminal groups. Changes in the infrared spectra were strongly strain-dependent, denoting the activation of different degradation pathways. Through the SEM analysis, the actual damages provoked by fungi were observed, including swellings, pits and furrows, bumps and partial exfoliations. Considering the rising concern about plastic disposal worldwide, the ability of these fungi to colonize PE and utilize it as carbon source is of great interest, as no pretreatments and pro-oxidant stimulants were needed.

Authors contributions

Federica Spina: Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. Maria Laura Tummino: Validation, Investigation, Writing – original draft. Anna Poli: Formal analysis. Valeria Prigione: Methodology, Formal analysis. Viktoria Ilieva: Methodology, Investigation. Piersandro Cocconcelli: Conceptualization, Funding acquisition. Edoardo Puglisi: Conceptualization, Methodology, Investigation. Pierangiola Bracco: Conceptualization, Writing – review & editing, Supervision. Marco Zanetti: Conceptualization, Funding acquisition, Supervision. Giovanna Cristina Varese: Conceptualization, Writing – review & editing, Funding acquisition, Supervision.

1. Introduction

Due to their chemical stability, good mechanical properties, low production costs and simple processability, plastic materials (basically composed by synthetic organic polymers) are key components in many manufacturing sectors, as packaging, clothing, construction, automotive, etc. (Akhbarizadeh et al., 2020; Thiounn and Smith 2020). The production trend of fossil-based plastics is constantly increasing, currently reaching 300–400 million ton per year (Ghatge et al., 2020), more than 80% of which is constituted by thermoplastic polymers as polyethylene (PE), polypropylene (PP), polyvinylchloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET) (de Souza Machado et al., 2018), the so-called

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commodity plastics (Kawecki et al., 2018). Although their favorable characteristics have surely caused strong benefits on the daily-life of global population, plastic materials are recalcitrant compounds to be degraded (Cruz Sanchez et al., 2020), mainly due to their chemical structure. In particular, PE, PP, PS and PVC have an extensive inert C—C backbone devoid of functional groups, thus resistant to hydrolysis (Inderthal et al., 2021) and to most chemical attacks, whereas PET, despite the presence of the ester group, possesses an aromatic ring that inhibits the degradation (Zhang 2015). The main-chain structure of these polymers can be broken down only via high-energy oxidation reactions (Inderthal et al., 2021). For these reasons, improper and indiscriminate disposal of plastics with their consequent accumulation in landfills and environment is a cutting-edge ecological concern (Cruz Sanchez et al., 2020; Thiounn and Smith 2020). The plastic recycling can represent one of the valid strategies to solve the issue, but the data related to recycling rate are not yet encouraging: for instance, only 10% and 6% of high-density polyethylene (HDPE) and low-density polyethylene (LDPE), respectively, are currently properly recycled (Thiounn and Smith 2020). This phenomenon lays its roots on the long-lasting thoughtlessness about the actual effect of plastic release in the environment. Despite the absence of direct toxicity of plastic polymers, the idea that plastics are harmless is long gone (Krueger et al., 2015). Mainly due to the ingestion, microplastic particles may negatively impact soil and water biota. The potential effects on aquatic organisms such as fishes (Gedik and Eryaşar 2020; Zhang et al., 2020), brine shrimp (Suman et al., 2020), medaka (Chisada et al., 2019) and oyster (Bringer et al., 2020) has been deeply documented. The terrestrial ecosystem is now showing not to be imperturbable: plastics can cause damages to snail (Chae and An 2020), silkworm (Parenti et al., 2020), springtail (Kim and An 2020), etc. Sediments, as well, are not avoiding the problem (Ziajahromi et al., 2018). Physical damages to the organisms are not the only way plastic particles may harm living organisms. Due to their high surface area and hydrophobicity, they can act as carriers of many pollutants (Fu et al., 2020). An active transportation route has been described for trace elements (Bradney et al., 2019; Prunier et al., 2019; Wang et al., 2020) and aromatic pollutants (Bellas and Gil 2020; Rodríguez-Seijo et al., 2019; Xu et al., 2020). How this affects higher trophic levels remains little explored, even though the actual transfer of microplastics among vertebrates or plants has found several evidences in terrestrial (Chae and An 2020; Guo et al., 2020) and aquatic (da Costa Araújo et al., 2020; Elizalde-Velazquez et al., 2020) ecosystems.

After more than half a century of widespread use and discharge of plastics, they are now recognized as an environment catastrophe, particularly due to their deliberate longevity and large-scale pollution. The persistency of plastic waste in the environment has triggered nature to face the problem, continuously adapting and evolving. Many microorganisms, ubiquitously present in aquatic and terrestrial ecosystems, are capable of turning plastic into their ecological niche, the so-called *plastisphere* (Amaral-Zettler et al., 2020). Both fungi and bacteria proved their efficacy in degrading recalcitrant molecules, including plastic, converting them into not-toxic compounds. Despite the various papers reporting bacteria-driven biodegradation, fungi are very promising organisms for PE degradation (Muhonja et al., 2018). Indeed, fungi are ubiquitous organisms, able to colonize all matrices (soil, water, air) and survive in a variety of natural and anthropized environments, where they help maintaining the ecosystem equilibrium (Anastasi et al., 2013). Their key factor for bioremediation purposes is the production of extracellular enzymes such as laccases, peroxidases and esterases, that are directly involved in the attachment of the PE surface and the consequent biodegradation (Wei and Zimmermann 2017). Thanks to their hyphal apparatus, they can also combine biochemical and physical actions, involving secondary metabolites as enzymes and biosurfactants (Kim and Rhee 2003; Sánchez 2020). However, these features are variable among fungi and the degradation skills are often strain dependent.

Indeed, the choice of the strain is not an easy step. Understanding how microbial communities of plastic-polluted environments are structured may ultimately have a major role on the effectiveness of the bioremediation strategy. Many studies have investigated the *plastisphere* by a metabarcoding approach (Esan et al., 2019; Puglisi et al., 2019; Lacerda et al., 2020); data supplied a clear view of this environment but they fail to provide a direct access to the microbial richness for any future exploitation purposes.

Thus far very few fungal species have been studied for their PE degrading ability, indicating the difficulty of obtaining fungal strains with outstanding degrading activity (Yuang et al., 2020). Ascomycetes such as *Aspergillus, Penicillium* and *Trichoderma* have been deeply investigated (Malachová et al., 2020, Ojha et al., 2017, Sáenz et al., 2019; Sowmya et al., 2014, 2015), whereas only recently research included other less known genera as *Cephalosporium* (Chaudhary and Vijayakumar, 2020) or *Fusarium* (Shi et al., 2020). Data about Basidiomycota or Mucoromycota are scarce with very few strains studied, i.e. *Phanerochaete chrysosporium* (Corti et al., 2012), *Bjerkandera adusta* (Kang et al., 2019), *Trametes versicolor* (Iiyoshi et al., 1998) and *Rhizopus oryzae* (Awasthi et al., 2017).

Most of the researches are using single strain without particular attention to the isolation source, *de facto* limiting the possibility of a successful treatment. Although PE wastes are remarkably stable during time, nature continues to adapt and evolve: fungal community dynamics can change due to the presence of plastic polymers, as noticed in the presence of PE (Esan et al., 2019; Wiedner and Polifka 2020) or even new generation biodegradable plastic as poly(butylene succinate-*co*-adipate) (PBSA) (Yamamoto-Tamura et al., 2020). This well-adapted biodiversity deserves further study, which will pave the way for future innovative strategies aimed at alleviating the environmental impacts of plastic materials. Moreover, in real ecosystems, additional factors may also help microorganism colonization and the consequent degradation of polymers (Awasthi et al., 2017). Polymer degradation is indeed subjected to a very complex combination of parameters, among which the physical-chemical properties of the polymer (density, molecular weight, crystallinity), the presence of additives and also the environmental conditions (i.e. exposure to light, heat and humidity) play a very significant role (Hakkarainen and Albertsson, 2004; Yuan et al., 2020).

In this context, the present paper is primarily focused on the development of an adequate methodology to study the PE-associated fungal community. These fungi may have adapted peculiar skills, potentially useful in environmental application: their role as degraders is, thus, investigated together with the occurred PE degradation by a multi-analytical approach.

2. Material and methods

2.1. Isolation of fungi

Sampling was carried out in an abandoned dumpsite in Northern Italy (Località Tavernelle, Fiorenzuola d'Arda, Piacenza Province). Approx. 42,000 tons of wastes were grounded for 10 years to a depth up to 4 m. Several persistent plastic materials were found on site. Plastic wastes were collected and identified as PE, as reported by Puglisi et al. (2019). The microbial community of PE wastes was isolated at the end of a long enrichment process. The

soil burial method was set up by two different strategies. The PE waste was directly inoculated in the soil (group A). In addition, the biofilm-forming microorganisms adhered to the plastic were extracted by sonication in a saline solution; 1 mL of solution was then inoculated in the presence of a commercial PE film and buried in soil (group B). In both cases, the presence or the absence of mineral oil was evaluated (2% mineral oil). Fifteen replicates were prepared for each condition. Trials were incubated at 24 °C. After 6 months, the biofilm-forming microbial extracts were prepared and used for a final liquid enrichment step: 1 mL of solution was inoculated in flasks in the presence of PE film and mineral medium.

After 1 month, 1 mL was used to prepared serial dilutions (10^{-2} – 10^{-4}). They were inoculated on plates with a modified Czapek medium with the addition of 1% mineral oil. Plates were incubated in the dark at 24 °C. Each colony was then transferred to Malt Extract Agar plates.

2.2. Identification of fungi

Fungal isolates were identified through a polyphasic approach, i.e. combining molecular and morphological traits. Briefly, fungi were initially classified through their morphological features using specific taxonomic keys; subsequently, specific genomic regions were amplified by PCR. Genomic DNA of each strain was extracted from mycelium grown in Malt Extract Agar (MEA: 20 g L^{-1} malt extract, 20 g L^{-1} glucose, 2 g L^{-1} peptone, 18 g L^{-1} agar) Petri dish using the NucleoSpin® Plant II kit (Macherey-Nagel). Amplification of specific genic regions occurred in a T100™ thermal cycler (Bio-Rad). Besides ITS region, actin, β-tubulin and elongation factor genes were amplified to identify *Cladosporium* spp., *Aspergillus* spp. and *Penicillium* spp. and *Fusarium* spp. and *Trichoderma* spp. strains (Poli et al., 2020).

PCR products were purified and sequenced at Macrogen Europe. Consensus sequences were obtained by using Sequencer 5.0 (Gene Code Corporation). Newly generated sequences were deposited in GenBank (accession numbers MK053578-MK053588, MK067041-MK067054, MK501840-MK501849, MK503137, MK503779-MK503786, MN962642-MN962649, MN963984-MN963986, MT413138, MT419360-MT419363).

2.3. PE solid screening

A primary solid screening was carried out to select fungal strains capable of growing in the presence of LDPE as sole carbon source. Low density polyethylene (LDPE, hereafter PE), Riblene® FM-50 (0.933 g cm^{-3}), was provided by Versalis S.p.A. PE granules were pulverized to obtain a powder (size <500 μm) in a Retsch SM 200 cutting mill under liquid nitrogen; the powder was then dried in a ventilated oven at 105 °C for 24 h.

Petri dishes of 6 cm diameter were prepared with agarized mineral medium (MM), where PE was the sole carbon source. The MM was a modified Czapek medium (Varjani Sunita et al., 2013). The PE powder was sterilized with 70% ethanol for 1 h, and then added to MM at 5 g L^{-1} and 10 g L^{-1}.

Strains were cultured on MEA plates at 24 °C for 7 days. A conidia suspension was prepared as described by Spina and collaborators (Spina et al., 2016), and inoculated in plates. Negative controls (MM plates without carbon source) and positive control (MM plates with 5–10 g L^{-1} glucose) were set up. The experiment was carried out in three replicates. Plates were incubated in the dark at 24 °C, and the colony diameters were periodically measured during the two weeks experiment. Data were expressed in term of growth percentage in comparison with the control with glucose.

Data were analyzed by means of Mann-Whitney test (means comparison) using SPSS 26.0 (SPSS for Windows, Chicago, IL, USA).

2.4. Biodegradation studies

2.4.1. Respirometry assay

A respirometry reactor was set up. Biodegradation yields were estimated by evaluating the CO_2 production as indicator of PE mineralization. The CO_2 released by fungi reacted with a NaOH solution (0.01 M, pH 8), causing the pH change. The pH indicator (bromocresol purple 0.05 g L^{-1}) allowed to correlate the pH variation with the color change. Reactors were prepared using a 100 mL sealed container with 45 mL of agarized MM (10 g L^{-1} of PE powder as sole C source) and a 3 mL glass vial containing 600 μL of bromocresol purple solution (Figure S1). Fungi were inoculated as previously described. Abiotic controls were prepared to verify the stability of system. After 10 and 30 days of incubation, 100 μL of the bromocresol purple solution were sampled and the absorbance at 590 nm was spectrophotometrically measured using Infinite M200 (TECAN Trading, Austria). A calibration curve allowed the correlation between the pH of the NaOH solution and the color of the pH indicator.

2.4.2. PE film degradation

Among fungi that, in the presence of PE, showed a comparable or even better growth than control, one representative for each species was selected. Thirteen fungi selected in the previous screening were tested. PE films, with a thickness of approximately 400 μm, were produced from PE pellets by hot pressing. Film portions (squares of 2 cm^2) were sterilized with 70% ethanol and air-dried. Samples were added onto each MM plate, following the methodology described by Koitabashi et al. (2012). The fungal inoculum was prepared as previously described. After 30 days, the PE films were washed with deionized water and, if necessary, a slight friction was applied to remove fungal mycelium.

Modifications to the PE structure were determined by comparing the vibrational features of pristine and biodegraded films. Therefore, Attenuated Total Reflectance - Fourier Transform Infrared (ATR-FTIR) spectra were recorded with a PerkinElmer Spectrum One spectrometer with a DTGS detector, equipped by a Universal ATR accessory, with a diamond/ZnSe crystal. Spectra were collected in the 4000-550 cm^{-1} range, with a resolution of 4 cm^{-1} and 8 scans/spectrum.

In order to detect the physical changes occurred on the surface, films were investigated by high-resolution Scanning Electron Microscopy (SEM), using a ZEISS EVO 50 XVP instrument with LaB_6 source, equipped with detectors for secondary and backscattered electrons collection. Before SEM analysis, films under examination were covered with a gold layer of ca. 20 nm of thickness to prevent charging (Bal-tec SCD050 sputter coater). SEM image acquisition was carried out by employing the secondary electrons detector and accelerating voltage ranging from 10 to 20 kV.

3. Results and discussion

3.1. Fungal biodiversity discovery

Despite the recent efforts to develop a more efficient management, plastic wastes still pose serious environment issues. In the nearby future, innovative eco-friendly solutions may lead to the sustainable clean-up of polluted sites, but the actual degrading agents have not been discovered yet. The present research targeted the fungal community of plastic wastes in a landfill soil by means of a culturomic approach, looking for fungi with promising degradation skills against recalcitrant plastics. The six months lasting enrichment was a powerful tool for the isolation of microorganisms from this extreme environment. A rich fungal biodiversity was brought to light: 95 strains belonging to 14 genera and 27 species

were isolated (Table S1). This rich microbial community is not often found in plastic contaminated environment. For instance, in previous reports, no more than 5–10 strains were isolated from soils collected from a plastic dumping site (Ojha et al., 2017) or municipal solid waste landfill rich in plastics (Das and Kumar 2014).

The long exposure was required by the high recalcitrance of PE, since shorter times can affect the isolation efficiency. For instance, Abraham et al. (2016) isolated only 4 fungi, but PE enrichment lasted only 15 days. Complex and stable polymers need long contact time. Soil burial of PVC took, instead, 10 months to isolate four fungi, i.e. *P. chrysosporium, Lentinus tigrinus, Aspergillus niger* and *Aspergillus sydowii* (Ali et al., 2014). On the contrary, biodegradable biopolymers are easier to handle, and microorganisms are faster to adapt. For instance, 67 Actinomycetes, 7 bacteria and 5 fungal isolates were obtained from agricultural soils with polylactic acid (PLA) after only 30 days (Penkhrue et al., 2015).

The presence of PE along the entire process led to the shaping of a peculiar mycobiome. The soil burial method helps highlighting the fungal community of both the surrounding soil and the actual PE-adhered one. The experiment was set up using directly the PE collected in the landfill (group A) or the microbial broth extracted from the wastes inoculated in the presence of commercial PE (group B). Significant differences were ascribable to the factors taken into account (e.g. inoculum method and presence of oil to stimulate the biofilm formation). The two groups were significantly different ($p < 0.0001$), with an average dissimilarity of 86.20%. The average intragroup similarity was low (14.66% and 23.37% for A and B, respectively), indicating that the species were heterogeneously distributed. Moreover, the presence of mineral oil significantly affected the developed community ($p < 0.0001$) in both groups (Figure S2). This finding is in agreement with other studies, where mineral oil or Tween 80 have enhanced the biofilm formation and biodegradation of PE, acting as modulators of hydrophobic interaction between the polymer and the cell wall (Devi et al., 2015).

Herein, most of the fungi belonged to Ascomycota, whereas only 2% were Mucoromycota. The absence of Basiodiomycota was not a surprise: despite few exceptions (Kang et al., 2019), they do not seem to possess the proper enzymatic pattern to compete in this ecological niche. The prevalence of Ascomycota in the plastic mycobiome found evidence in the literature (Raghavendra et al., 2016). Moreover, the fungal community was here dominated by the genera *Fusarium, Purpureocillium* and *Aspergillus* that covered almost 70% of isolated fungi (Fig. 1). Likewise, *Fusarium* and *Aspergillus* were the most common indigenous fungi in plastic garbage of landfill sites from different Indian regions (Raghavendra et al., 2016). *Aspergillus* and *Cladosporium* were the most abundant genera associated with marine plastics, being ubiquitously present in samples of different origin (Lacerda et al., 2020).

The role of plastic wastes as vectors for microbial pathogens has been poorly understood, and mostly investigated in marine ecosystems (Lacerda et al., 2020). The isolation of several representatives of *Scedosporium/Pseudoallescheria* genera (namely *P. boydii, S. apiospermum, S. aurantiacum, S. dehogii*) poses serious concerns about the safety and the disposal of landfill soil and its percolate. *Scedosporium* spp. are indeed emerging pathogens that could affect both immunocompromised and immunocompetent people (Luna-Rodríguez et al., 2018).

3.2. PE solid screening

All isolated fungi have been in contact with PE for six months and have been isolated in a not-generic medium. They allegedly belonged to the *plastisphere* because capable of exploiting such polymer as carbon source. Since this feature may, of course, be differently expressed by each strain, one of the goals of the present study was a deeper investigation. The solid screening showed that most of fungi (97%) displayed the ability to grow in presence of PE as sole carbon source in at least one cultural condition (5 g L^{-1} or 10 g L^{-1}). The only exceptions were *Chrysosporium lobatum*, *Cladorrhinum bulbillosum*, *Cladosporium aggregatocicatricatum* and *Mucor circinelloides* that did not grow in the presence of PE. Since the negative control indicated a null growth, fungi demonstrated to be active in transforming and using the PE as nourishment.

The PE concentration is a well-known limiting factor. Although many researches commonly used low PE concentrations (Nwogu et al., 2012), in the present study, some strains tolerated the exposition to high PE content. Eight out of 95 fungi (all belonging to *Fusarium solani* and *Fusarium oxysporum*) grew better at 10 g L^{-1} of PE ($p < 0.05$). *F. solani* and *F. oxysporum* showed the highest difference of growth, compared to positive control (+32% and +24%, respectively), indicating a strict correlation between the growth rate and PE as C source.

The 26.3% of tested fungi (25 out of 95) grew regardless the PE concentration. Fig. 2 indicates the strains with a comparable growth in the presence of 5 and 10 g L^{-1} PE. They mostly belonged to *Fusarium* and *Aspergillus* genera, but a common behavior was observed by *Penicillium chrysogenum, Purpureocillium lilacinum, R. oryzae, S. aurantiacum* and *Trichoderma brevicompactum*. Likewise, literature reports that *F. oxysporum, Aspergillus fumigatus, Lasiodiplodia crassispora, A. niger, Penicillium* sp. and *Trichoderma harzianum* can degrade PE, even producing a clearance zone around the growing culture (Raghavendra et al., 2016). Five *Trichoderma* isolates were screened in mineral medium with emulsified PE powder, showing an increased colony size by three of them (Hikmah et al., 2017). In general, members of the genus *Aspergillus* are known to degrade plastic polymers as PE (Lacerda et al., 2020; Pramila and Ramesh, 2011; Sangale et al., 2019). *Fusarium* strains have been already associated with polymer degradation as LDPE, PET, poly(ε-caprolactone), and the involved enzymatic pattern has been partially described (Hasa et al., 2007; Nimchua et al., 2007; Shi et al., 2020; Wei and Zimmermann 2017). Noteworthy, in the present study, the ability of *P. lilacinum* and *S. aurantiacum* to transform PE has been reported for the first time.

3.3. PE degradation trials

The activity of the microorganisms against PE was confirmed by the respirometry assay. Biodegradation was evaluated by following CO_2 production, as an indicator of PE powder mineralization. Respirometry tests were run with 13 selected strains that showed good performances in the screening tests. Table 1 lists the pH of the indicator solutions after 10 and 30 days. The amount of CO_2 released from the control remained stable, confirming the reliability of the technique. After 10 days, all fungi produced a significant amount of CO_2 that caused a consistent acidification of the solution. At the beginning, they all reached pH values below 5. As regards 6 strains (*P. lilacinum, F. oxysporum, Aspergillus flavipes, T. brevicompactum, P. chrysogenum* and *F. solani*), the acidification increased with time (i.e. *P. lilacinum* and *F. oxysporum* up to pH 3.6 after 30 days), indicating a progressive PE transformation. Since the microbial attack usually begins with a colonization and adhesion to the surface, ATR-FTIR and SEM are useful tools to observe the effects of the fungal contact.

3.3.1. 3,1 ATR-FTIR analyses

Some examples of ATR-FTIR spectra of PE films after incubation are reported in Fig. 3, showing the chemical modifications made by selected fungi: *P. chrysogenum*, *F. oxysporum*, *T. brevicompactum*, *P. lilacinum* and *F. falciforme*. Fig. 3A gives an overview of the entire PE spectra after biodegradation, whereas Fig. 3B, C and 3D show

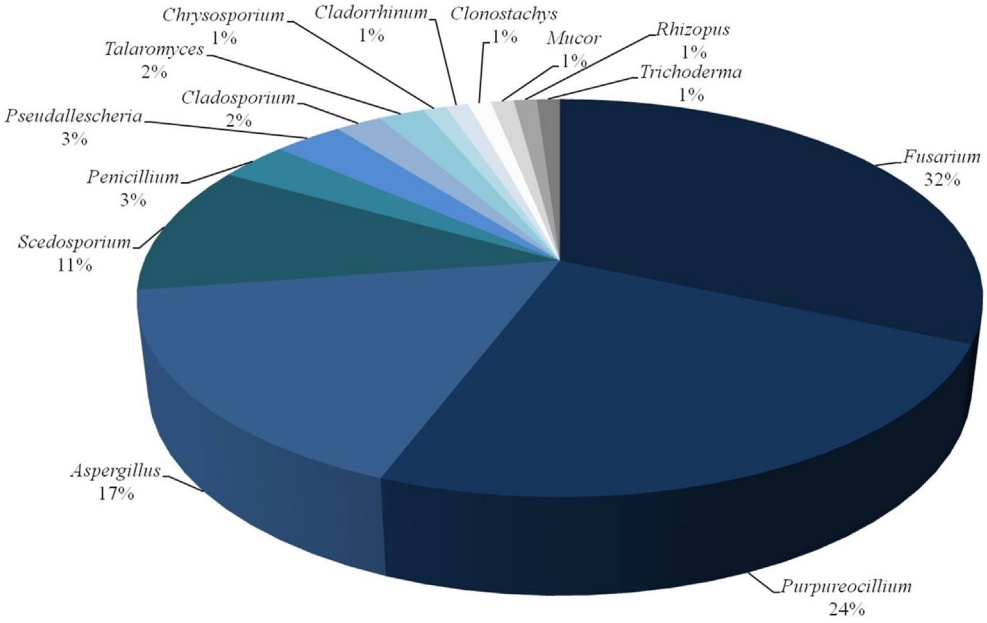

Fig. 1. Abundance percentage of the identified genera.

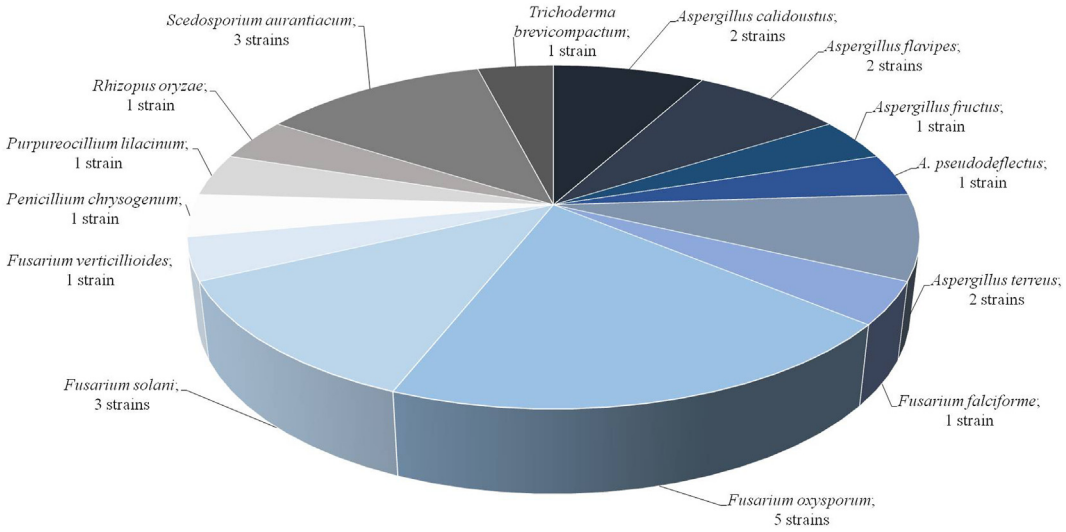

Fig. 2. List of fungi that comparably grew in the presence of PE at different concentration (5–10 g L^{-1}).

Table 1
PH values measured in the respirometry trials after 10 and 30 days of treatment.

strains	10 days	30 days
Aspergillus calidoustus	4.8	6.4
Aspergillus flavipes	5.0	4.0
Aspergillus fructus	5.0	4.9
Aspergillus pseudodeflectus	5.0	5.1
Aspergillus terreus	4.5	5.5
Fusarium falciforme	4.6	4.6
Fusarium oxysporum	4.3	3.6
Fusarium solani	4.8	4.3
Fusarium verticillioides	4.6	4.6
Penicillium chrysogenum	5.2	4.2
Purpureocillium lilacinum	4.5	3.6
Rhizopus oryzae	4.8	4.6
Trichoderma brevicompactum	4.6	4.1
control	7.4	7.3

specific ranges of wavenumbers comprising characteristic signals. An abiotic control sample, constituted by a PE film left in agarized mineral medium without fungal inoculum, was used as a reference.

As previously described, all the samples display the typical IR absorptions of PE (Charles and Ramkumaar 2009; Gulmine et al., 2002; Jung et al., 2018): strong bands at 2915 and 2848 cm^{-1} due to $\nu_{as}(CH_2)$ and $\nu_s(CH_2)$, respectively; in-plane $\delta(CH_2)$ at 1472 and 1463 cm^{-1}; $\delta(CH_3)$ at 1378 cm^{-1} close to three signals at 1368, 1353 and 1305 cm^{-1} attributed to $\omega(CH_2)$; the last absorption at 718 cm^{-1} is ascribed to $\rho(CH_2)$.

In the 1400-1000 cm^{-1} region (Fig. 3B), for PE incubated with *P. lilacinum* and *F. falciforme*, the intensity of signals between 1330 and 1390 cm^{-1} increased and the absorbance ratio between CH_3 (1378 cm^{-1}) and CH_2 (1366 cm^{-1}) was reversed with respect to the control sample. In general, these signals and their relationship are indicators of the branching degree of PE (Gulmine et al., 2002) and

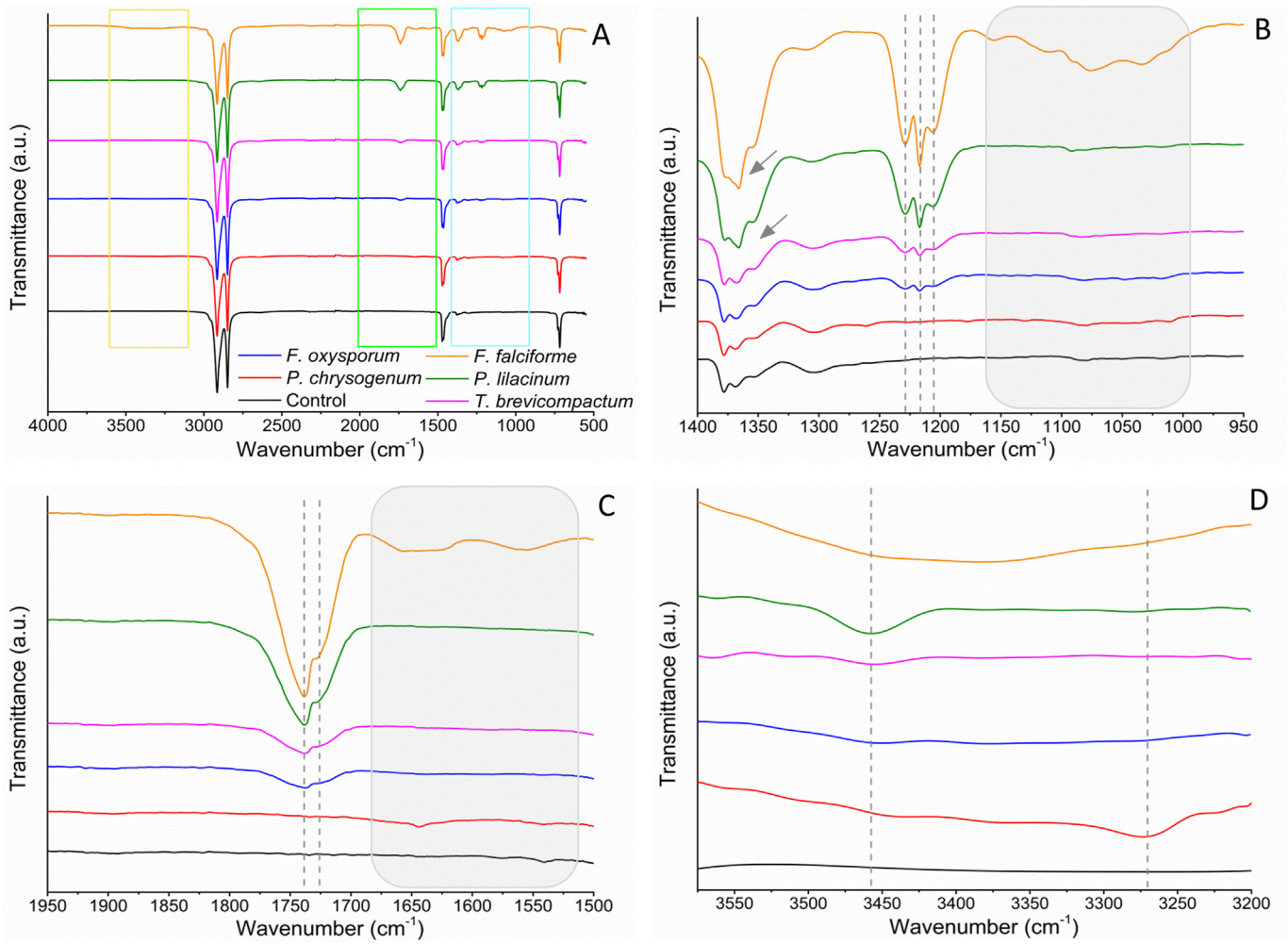

Fig. 3. ATR-FTIR spectra of PE films after 30 days of incubation with *Penicillium chrysogenum* (red line), *Fusarium oxysporum* (blue line), *Trichoderma brevicompactum* (magenta line), *Purpureocillium lilacinum* (green line), *Fusarium falciforme* (orange line) plus the control sample (black line). Different ranges are shown: (A) 4000-550 cm^{-1}, (B) 1400-950 cm^{-1}, (C) 1950-1500 cm^{-1} and (D) 3575-3200 cm^{-1}. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

have been already employed to distinguish LDPE and HDPE (Jung et al., 2018). The decrease in the relative amount of methyl groups after the contact with fungi suggests that biodegradation occurred at the expenses of methyl terminal groups. The cleavage of PE chains with the breakage of C–C bonds have been similarly triggered by different organisms (Kundungal et al., 2019; Sen and Raut 2015; Sheik et al., 2015).

The absorption band centered on 1220 cm^{-1}, composed by three contributions, is evidenced in Fig. 3B: it is attributed to ν(C–O–C) and indicates the occurrence of the oxidation process (Muhonja et al., 2018). This correlates with the absorption at 1738 cm^{-1} and the shoulder at 1722 cm^{-1}, related to ν(C=O) of ester and keto-groups, respectively (Fig. 3C) (Balasubramanian et al., 2010). The oxidative degradation is a convenient pathway for polymers devoid of easily hydrolysable groups, as PE (Yuan et al., 2020), and it is promoted by microbial enzymes to create functional groups that improve polymer hydrophilicity and, consequently, the biodegradability (Shah et al., 2008). An increase in the amount of carbonyl groups in LDPE was also noticed in the presence of *Aspergillus nomius* (Abraham et al., 2017) and *R. oryzae* (Awashi et al., 2017), a sign that has been directly associated to peroxidase attack (Sudhakar et al., 2007). Devi et al. (2015) noticed an initial increase of the carbonyl content that decreased later, after prolonged incubation with *Aspergillus tubingensis* and *Aspergillus flavus*. The same behavior has often been observed concluding that biodegradation decreases the amount of carbonyl groups, in contrast with abiotic degradation caused by other environmental factors (Albertsson et al., 1987).

Residues of biomass and/or agar-based medium are mainly detectable by peaks framed in the grey areas of Fig. 3B and C: some vibrations related to proteins, nucleic acids and carbohydrates are mostly present within the ranges 1000-1150 cm^{-1} and 1540-1650 cm^{-1} (Lecellier et al., 2015; Naumann 2015). In the same regions, some overlaps consequent to PE degradation cannot be ruled out, as in particular, the formation of terminal C=C (*ca.* 1650 cm^{-1}) (Balasubramanian et al., 2010; Puglisi et al., 2019) and C–O of alcohols (1020-1100 cm^{-1}) (Muhonja et al., 2018).

Fig. 3D displays a significant enlargement of high wavenumber region, showing the signals at *ca.* 3270 cm^{-1} and above 3450 cm^{-1} together with the broad band centered around 3380 cm^{-1} (visible in the case of *F. falciforme*), which are associated to ν(NH) of the proteins residue and to ν(OH). For the latter, different shifts can be the result of the differences in hydroxyl group origin, namely PE oxidation (Kundungal et al., 2019; Puglisi et al., 2019), residual biomass (de Oliveira 2020; Lecellier et al., 2015) and/or physisorbed water (Sheik et al., 2015).

Focusing on the different activities of various strains under examination, *P. chrysogenum* (Fig. 3) exemplifies the behavior of those

fungi that caused negligible or, at least, mild modifications of PE macromolecular structure. Indeed, the FTIR spectra of PE treated with *Aspergillus pseudodeflectus*, *A. flavipes*, *Aspergillus calidoustus*, *A. terreus*, *Fusarium verticillioides*, *P. chrysogenum*, *R. oryzae*, *F. solani* did not show remarkable variations with respect to the control (Figure S3). On the contrary, the peaks related to biodegradation reactions (i.e. oxygenated species) are evident in the presence of *F. oxysporum*, *T. brevicompactum*, *P. lilacinum* and *F. falciforme* (Fig. 3), whereas *Aspergillus fructus* led to an intermediate degree of degradation (small peak at 1748 cm^{-1} in Figure S3).

3.3.2. 3,2 SEM investigation

Analogously to ATR-FTIR, SEM showed various PE surface modifications caused by fungi with respect to the control sample that is characterized by a smooth and regular morphology (Fig. 4A). A selection of SEM images is reported in Fig. 4. The biodegradation of PE was evident: cavities, grooves and a flaking structure appeared on the surface of the film. Moreover, despite the pre-washing, fungal hyphae and conidia were still present on the surface, indicating the strong adhesion of the mycelium on PE.

P. chrysogenum showed a scanty and localized growth on the PE film (only on the bottom side), leaving hyphae and spores, as it can be seen in Fig. 4B. In agreement with previous observations (Sáenz et al., 2019; Sheik et al., 2015), *F. oxysporum* widely colonized both sides of PE film, forming bumps and partial exfoliations (Fig. 4C). *T. brevicompactum* exhibited a widespread colonization (Fig. 4D) probably associated to the excretion of extracellular biofilm production (Mathur et al., 2011). However, it produced minor but still

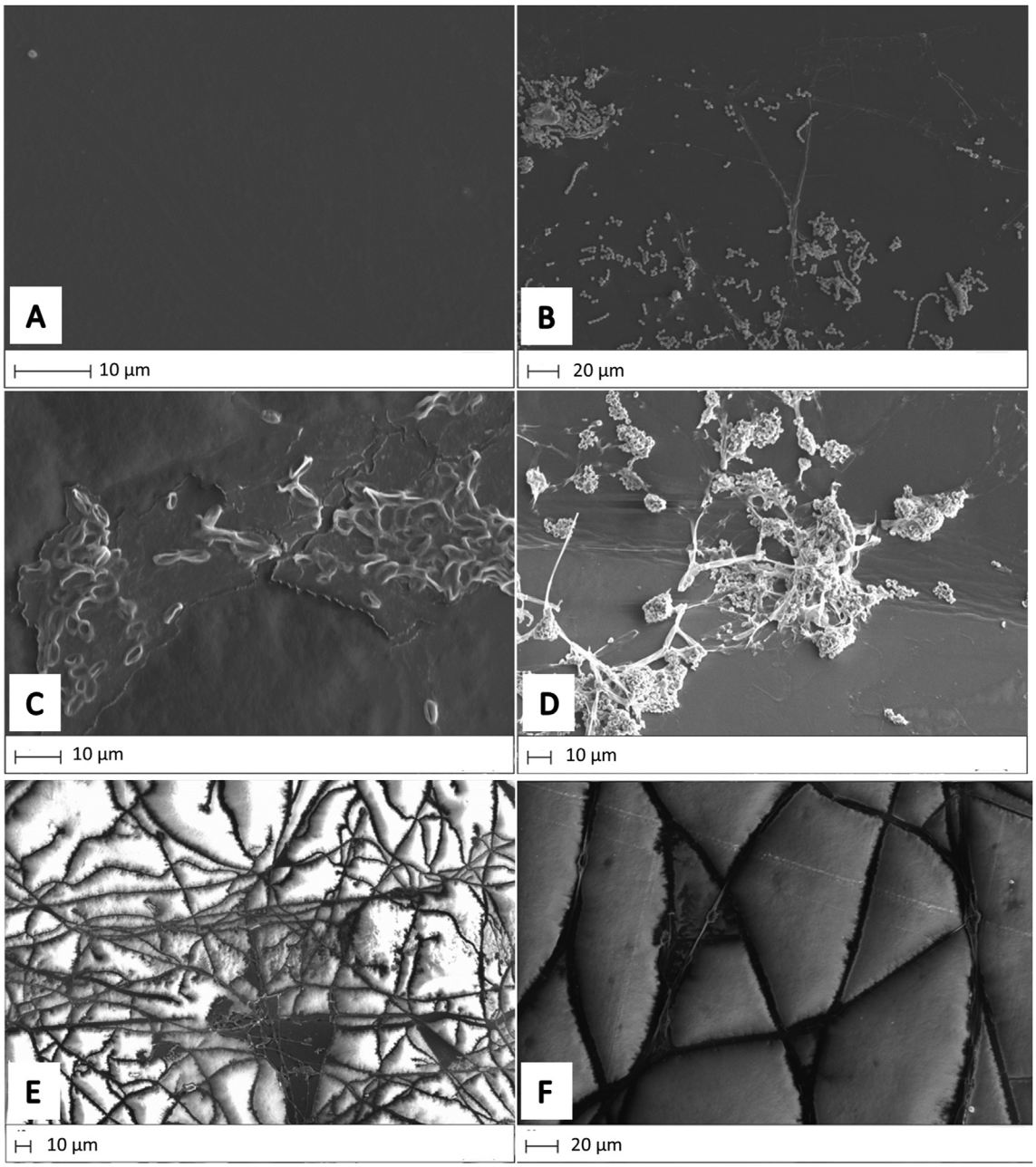

Fig. 4. SEM images at high magnification for PE films subjected to fungal activity, control film (A) *P. chrysogenum* (B), *F. oxysporum* (C), *T. brevicompactum* (D), *P. lilacinum* (E) and *F. falciforme* (F). The differences in the magnification allowed to evidence the specific superficial features.

perceivable morphological alterations, as ripples on the PE surfaces. *P. lilacinum* and *F. falciforme* provoked significant damages on PE surfaces, namely swellings, pits and furrows, reflecting the presence and/or the remaining imprints of the fungal hyphae (Fig. 4E–F).

As for the other strains (Figure S4), it is possible to group them according to the observed modifications: (i) no remarkable spoilage by *R. oryzae*, (ii) mild damages by *A. fructus*, *A. flavipes* and *A. terreus*, (iii) noticeable swelling, cracks and hyphae-derived furrows by *A. pseudodeflectus, F. verticillioides*, *A. calidoustus* and *F. solani*.

3.3.3. 3,3 degradation assessment summary

The above-described outcomes are in agreement with the general model that describes the beginning of polymer biodegradation as a superficial erosion process (Gajendiran et al., 2016): the microorganisms colonize the surface and adhere, with the production of extracellular enzymes and other secretions to form a biofilm (Koutny et al., 2006; Sen and Raut 2015; Sheik et al., 2015). This critical first step is fundamental to trigger the microbial biodegradation (Mathur et al., 2011). Moreover it can overcome the hurdles of both the dimensions of enzymes that are too large to deeply penetrate into the material bulk (Gajendiran et al., 2016) and the size of non-degraded macromolecules, too large to pass through cellular membranes (Koutny et al., 2006; Sen and Raut 2015). The production of the biofilm can also counteract the hydrophobicity of polyethylene and help the microorganisms to survive in a low-nutrient environment using the solid substrate (Ojha et al., 2017; Sen and Raut 2015; Shah et al., 2008; Yuan et al., 2020).

F. oxysporum, F. falciforme and *P. lilacinum* can be identified as the most promising strains as they all distinctly affected the PE film morphology. The strongest oxidation phenomena assessed by FTIR was caused by *F. falciforme* and *P. lilacinum*, while *F. oxysporum* and *P. lilacinum* exhibited the greatest capability to mineralize PE, as indicated by the strong acidification obtained in the respirometry tests. Fungi seemed to first oxidize the polymer, but only in some cases, this led to its actual mineralization. When the degradation strongly proceeds (as for *F. oxysporum*), the degraded fraction of PE is almost totally mineralized and the intermediate oxidized PE can be undetectable to FTIR analyses.

A. fructus showed a moderate activity, confirmed by respirometry test, FTIR analysis and SEM images. The other six strains (*A. pseudodeflectus*, *A. flavipes*, *A. calidoustus*, *A. terreus*, *F. verticillioides*, and *F. solani*) mostly affected the PE film topography, without relevant chemical modification of the macromolecular structure. *T. brevicompactum* also showed a partial degradation: it abundantly colonized the PE films and led to the appearance of ester carbonyl, but provoked mild changes in the smoothness of the PE surfaces. These data did not fully agree with literature, probably due to the intraspecific variability among strains. In some studies, *Aspergillus* strains have shown high degradation skills (Muhonja et al., 2018; Sáenz et al., 2019). *Trichoderma* sp. damaged LDPE membranes producing pores on the surface (Hikmah et al., 2018). However, *Trichoderma hamatum* had a very low impact on LDPE and HDPE, unless the films were previously γ- or UV-irradiated (Malachová et al., 2020). Additional studies are necessary to better highlight the fungal performances, for example by setting longer exposure time. *Aspergillus niger* and *A. terreus* created cracks on LDPE films, but over a period of 77 days (Sáenz et al., 2019).

4. Conclusion

Thanks to their morphological and physiological features, fungi confirmed to be ubiquitous organisms, able to colonize atypical matrices as PE surface. The mycobiota isolated from the plastic contaminated site denoted a diversified fungal community: the adaptation to this anthropized environment has led to peculiar metabolic skills. Four fungi (*A. fructus, F. falciforme, F. oxysporum* and *P. lilacinum*) were very active against PE, extensively colonizing the films, causing significant damages and triggering oxidative transformation of the polymer. These fungi have demonstrated to possess exceptional metabolic features that may pave the road for their exploitation as bioremediation agents. Further studies will allow useful information about the management of wastes and their possible re-use.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.116548.

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