



Biochar and hydrochar from waste biomass promote the growth and enzyme activity of soil-resident ligninolytic fungi



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ABSTRACT

Biochar (BC) and hydrochar (HC) are carbonaceous products obtained through, respectively, pyrolysis and hydrothermal carbonization processes of biomass. Both materials are multi-functional soil amendments. Ligninolytic fungi are primary decomposers of recalcitrant lignocellulosic material in nature through their extensive hyphal network and enzymes. In this work, two BC samples from red spruce pellets (BC_{SP}) and grapevine pruning residues (BC_{GV}) and two HC samples from urban pruning residues (HC_{UP}) and the organic fraction of solid urban wastes (HC_{SU}) were tested at concentrations of 0.4% and 2% (w/v) on the growth and enzyme activity of *Trametes versicolor*, *Pleurotus ostreatus* and *Pleurotus eryngii*. In all treatments with the lower concentration, BC and HC significantly stimulated fungal growth (up to about 90% increase for HC_{SU} on *T. versicolor*), whereas at the higher dose some inhibition was observed on *T. versicolor* by BC_{SP} and *P. ostreatus* by BC_{SP}, BC_{GV} and HC_{UP}. The two materials, especially HC, at both doses noticeably increased the activity of laccase from *T. versicolor* and *P. eryngii*, up to 21 and 13 times, respectively, for HC_{UP} compared to controls. The activity of manganese peroxidase from *P. ostreatus* was also greatly stimulated by BC and HC, especially when added at the higher concentration. The overall results obtained in this study suggest potential benefits for ligninolytic fungi from the presence of these materials in soil at adequate dose of application.

1. Introduction

Biochar (BC) and hydrochar (HC) are solid materials obtained through pyrolysis and hydrothermal carbonization of biomass, respectively. The two processes are currently being investigated as novel recycling techniques that allow the carbon neutral/negative transformation and recycling of waste materials of diverse nature, such as tree pruning residues, corn stalks, chicken manure and the organic fraction of solid urban waste. While BC is widely recognized for carbon sequestration in soil, HC is a more recently introduced organic amendment. Both BC and HC can be considered as multi-functional soil amendments that can influence both physical and chemical properties of soil. Although BC is widely considered resistant to biodegradation, its use as carbon source by fungi is still an open question. Recent studies have demonstrated the presence in BC of bioavailable organic components like hydrophilic compounds and thermally labile fractions (Rombolà et al., 2016). BC from different feedstock and production techniques was found to contain adsorbed volatile organic compounds (Spokas et al., 2011), polycyclic aromatic hydrocarbons (Buss et al., 2015) and numerous potentially toxic

elements (Singh et al., 2010). The production conditions of HC, i.e., at temperatures lower than those requested for pyrolysis and wet feedstock, can better mimic the natural humification process and lead to materials that might resemble peat in terms of properties and applications (Álvarez et al., 2017). HC is generally considered more easily decomposable, owing to fewer aromatic structures and higher percentage of labile carbon content, compared to BC (Cao et al., 2011; Gascó et al., 2018). Further, also HC has shown the presence of components like adsorbed volatile organic compounds (Becker et al., 2013) and organic and inorganic contaminants (Jandl et al., 2013; Chakrabarti et al., 2015). In addition, for both materials, differences in feedstock and production parameters can greatly affect the properties of the final product and its impact on soil (Lehmann and Joseph, 2009; Hitzl et al., 2015).

Fungi of the Phylum Basidiomycota are critical in the cycling of nutrients in soil as they play the role of plant debris decomposers (Magan, 2008). In particular, white rot fungi are decomposers of recalcitrant lignocellulosic material for their ability to initiate the depolymerization of lignin and maintain the carbon cycle in soil (Barron, 2003; Lundell et al., 2010). This specific feature is the result of the combination of their

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extensive hyphal network and their enzymatic activity. White rot fungi mainly secrete two types of extracellular ligninolytic enzymes to depolymerize lignin, which are phenol oxidases (mainly laccases) and peroxidases (lignin peroxidase, Mn peroxidase, versatile peroxidase) (Schmidt-Dannert, 2016). Besides their role in soil fertility, and because of the low substrate specificity of their ligninolytic enzymes, some white rot basidiomycetes have been also used for decontamination purposes, e.g. decontamination of wastewater from phenolic endocrine disruptors (Loffredo et al., 2012, 2013; 2016; Traversa et al., 2012), dyes (Anastasi et al., 2010; Kalpana et al., 2011; Malachova et al., 2013), mycotoxins (Branà et al., 2017) and numerous other environmental pollutants, including polycyclic aromatic hydrocarbons and pesticides (Kim and Song, 2003; Davila-Vazquez et al., 2005; Rodríguez-Rodríguez et al., 2010; Cerrone et al., 2011; Loffredo et al., 2012, 2013, 2016).

Interaction of BC and HC with soil microorganisms, including fungi, is inevitable once these materials are incorporated into the soil as amendments. Lehmann et al. (2011) reviewed the impact of BC on soil biota, whereas other authors investigated the effects of BC on specific fungal groups or species, such as arbuscular mycorrhizal fungi (Warnock et al., 2007) and phytopathogenic fungi (Graber et al., 2014). Although white rot fungi are critical for processes occurring in soil environment, only a limited number of studies can be found in the literature about the impact of BC or HC on these fungi. Some investigations concern BC impact on colonization (Ascough et al., 2010), abundance (Jin, 2010), respiration (Gibson et al., 2016) and bio-depolymerization activity (Placido et al., 2016) of white rot species. Furthermore, more recently, some studies have described the combined use of BC and white rot fungi for remediation purposes (García-Delgado et al., 2015; Loffredo et al., 2012, 2013, 2016). In particular, the work of García-Delgado et al. (2015) focused on the involvement of enzymatic activity of the BC-associated fungi in bioremediation.

The number of published works on the impact of HC on soil-resident fungi is very limited and undoubtedly lower than that of BC on the same topic. Microbial community shift, including fungal species (Rex et al., 2015) and performance of arbuscular mycorrhizal fungi (Salem et al., 2013) in the presence of HC were the only two aspects addressed by scientists to date. Recent findings indicate that changes in the functionality of microbial community may occur as a consequence of BC and HC application to soil, and such changes would eventually have also a relevant impact on soil fertility, especially when ligninolytic fungi are involved. Given the importance of this matter, there is a clear need for further investigation that consider different feedstock in BC and HC production, different white rot fungal species, but also the possibility to combine the two amendments at adequate rates.

Therefore, the objective of this study was to investigate comparatively the impact of two BC samples, from spruce pellets and grapevine pruning residues, and two HC samples, from urban pruning residues and the organic fraction of solid urban wastes, on the growth and enzyme activity of *Trametes versicolor*, *Pleurotus ostreatus* and *Pleurotus eryngii*. These species of white-rot fungi were chosen because of their well-documented and high efficient ligninolytic activity and their cosmopolitan distribution.

2. Materials and methods

2.1. Biochar, hydrochar and fungal strains

BC samples were produced starting from red spruce pellets (BC_{SP}) and grapevine pruning residues (BC_{GV}) and supplied by Blucomb s. r.l., Udine, Italy. BC production was obtained by pyrolytic micro-gasification method with a residence time of 3 h and a peak temperature of 550 °C followed by dry cooling. HC samples, whose feedstocks were urban pruning residues (HC_{UP}) and the organic fraction of solid urban wastes (HC_{SU}), were produced and provided by Ingelita Italia s. r.l., Lucca, Italy. HC was produced using a hydrothermal carbonization chamber operating in the range of 180–210 °C with pressure between 10 and 20 bars.

Before starting the experiments, BC and HC samples were air-dried, ground with mortar and pestle and 0.5-mm sieved. A multianalytical characterization of the materials used in this work was performed by Taskin et al. (2019).

Isolates of *Trametes versicolor* (L.:Fr.) Pilát (CBS 114372) and *Pleurotus ostreatus* (Jacq.) P. Kumm. (CBS 145.22) were purchased from the Centraal Bureau voor Schimmelcultures (CBS-KNAW), Utrecht, The Netherlands. The isolate of *Pleurotus eryngii* (DC.) Quél. (ITEM 13681) was obtained from the culture collection of the Institute of Sciences of Food Production (ITEM Collection, <http://www.ispa.cnr.it/Collection/>), Bari, Italy. Fungal cultures were grown on potato dextrose agar (PDA, Oxoid, 4% w/v) in Petri dishes in the dark at 20 ± 1 °C for *T. versicolor* and *P. ostreatus*, and 25 °C ± 1 °C for *P. eryngii*. Fungal inoculum used for the experiments was a 4-mm radius mycelial disk excised from the growing margin of young colonies on PDA.

2.2. Assays on fungi

The impact of BC and HC samples on the three fungi was evaluated in two consecutive sets of experiments using the same experimental plates for fungal growth assessment and for enzyme activity assays. Briefly, aqueous suspensions of PDA (Oxoid, 4% w/v) were prepared and supplemented or not (control) with each BC and HC, separately, at concentrations of 0.4% (BC_{SP}L, BC_{GV}L, HC_{UP}L, HC_{SU}L) and 2% (BC_{SP}H, BC_{GV}H, HC_{UP}H, HC_{SU}H). The two doses were selected on the basis of recommended field application rates for BC (8–50 t ha⁻¹) reported in literature (Major, 2010). Suspensions were then sterilized in autoclave at 121 °C for 15 min. Aliquots of 20 mL were taken with a pipette from each medium kept under continuous stirring, transferred into 9-cm diameter Petri dishes and allowed to cool at room temperature under sterile conditions.

2.2.1. Growth assays

The fungi were inoculated in the centre of the plate under axenic conditions and the dishes were randomly placed in an incubator in the dark at a constant temperature of 21 ± 1 °C for *T. versicolor* and *P. ostreatus*, and at a temperature of 25 ± 1 °C for *P. eryngii*. Starting from 24 h after incubation, at sampling times depending on the growth rate of the fungus, radial growth of the mycelium (in mm) was measured until the fungus reached approximately the border of the plate at least in one Petri dish. All experiments were replicated five times.

2.2.2. Enzyme activity assays

After the end of growth experiments, the Petri dishes from treatments and controls were left for one week in an incubation chamber in the same conditions adopted for growth assays. After this period, each Petri dish was completely covered with mycelium, and three replicates were selected randomly and used for the enzyme activity assay.

Oxidative enzymes were extracted from solid growth medium using a procedure adapted from Berry et al. (2014). Briefly, ten PDA plugs of 8-mm diameter and a total weight of 3 g were removed from each plate along the colony radius, from the center (inoculum point) to the edge of the colony in every direction, using a sterile cork borer. The plugs were placed into 50 mL centrifuge tubes and 6 mL of phosphate buffer at a concentration of 100 mM at pH 7.3 was added. Successively, the samples were homogenized using an Ultra-Turrax® T 25 disperser (IKA-Werke GmbH & Co. KG, Staufen, Germany) equipped with 10 mm polycarbonate disperser blade, at 25000 rpm and filtered through Whatman® (Maidstone, UK) Grade 4 filter paper. Filtrates obtained were immediately placed in a refrigerator at 4 °C and analyzed on the same day. In addition, plugs from not inoculated plates were also analyzed to check any interference of BC and HC components in the enzyme assays. Laccase (EC 1.10.3.2) and manganese-dependent peroxidase (MnP) (EC 1.11.1.13) activities in the filtrates were determined spectrophotometrically by using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) and MnSO₄ as substrate, respectively. Activity of laccase was

determined by following the oxidation of ABTS at a concentration of 0.2 mM in sodium acetate buffer (pH 4.5) at concentration of 100 mM at a wavelength of 420 nm ($\epsilon = 36000 \text{ M}^{-1} \text{ cm}^{-1}$). MnP activity was assayed through the oxidation of MnSO_4 at a concentration 1 mM in sodium malonate buffer (pH 4.5) at a concentration of 50 mM and H_2O_2 at concentration of 0.1 mM at a wavelength of 270 nm ($\epsilon = 11590 \text{ M}^{-1} \text{ cm}^{-1}$). Enzyme activity was expressed as enzyme international units (IU). One IU is defined as the amount of enzyme producing 1 μmol of product per minute under the assay conditions used (García-Delgado et al., 2015).

2.3. Statistical analysis

All results obtained were statistically analysed by one-way analysis of variance (ANOVA) at 95%, 99% and 99.9% confidence levels. The means of treatments were statistically compared by the least significant differences (LSD) test using CoStat Statistical Software (Version 6400, CoHort Software Monterey, CA, USA).

3. Results

3.1. Growth assays

3.1.1. Biochar

The effects of BC samples on radial mycelial growth of *T. versicolor*, *P.*

ostreatus and *P. eryngii* at the two concentrations adopted are presented in Fig. 1. In general, during the entire experimental period, the presence of each BC at the lower dose caused a relevant stimulation ($P \leq 0.001$) of the growth of the fungi examined, with increases generally between 50% and 70% compared to control. The only exception was BC_{GvL} which did not alter the growth of *P. ostreatus* compared to the control (Fig. 1d). On the other hand, the presence of BC at the higher dose produced differentiated effects on the fungi depending on BC type and fungal species. A significant growth inhibition appeared during the experimental period in treatments with BC_{SpH} on *T. versicolor* and, to a lesser extent, on *P. ostreatus*, even if this effect was absent at the end of experiments (Figs. 1a and 1c). BC_{GvH} seemed do not affect the growth of *T. versicolor* initially, whereas a significant stimulation was evident in successive samplings, with increases of mycelial radius of 28% and 44% at 120 h and 144 h, respectively, compared to the control (Fig. 1b). Moreover, BC_{GvH} showed a slight inhibition of *P. ostreatus* growth that was reduced by a maximum of 24%, compared to the control, after 48 h from inoculation (Fig. 1d). Differently from the other two fungi, along the whole experiment, *P. eryngii* growth was always promoted by both BC_{SpH} and, especially, BC_{GvH}, with a maximum increase of about 70%, with respect to the control (Figs. 1e and 1f).

3.1.2. Hydrochar

Effects of HC samples on the radial mycelial growth of the three fungi

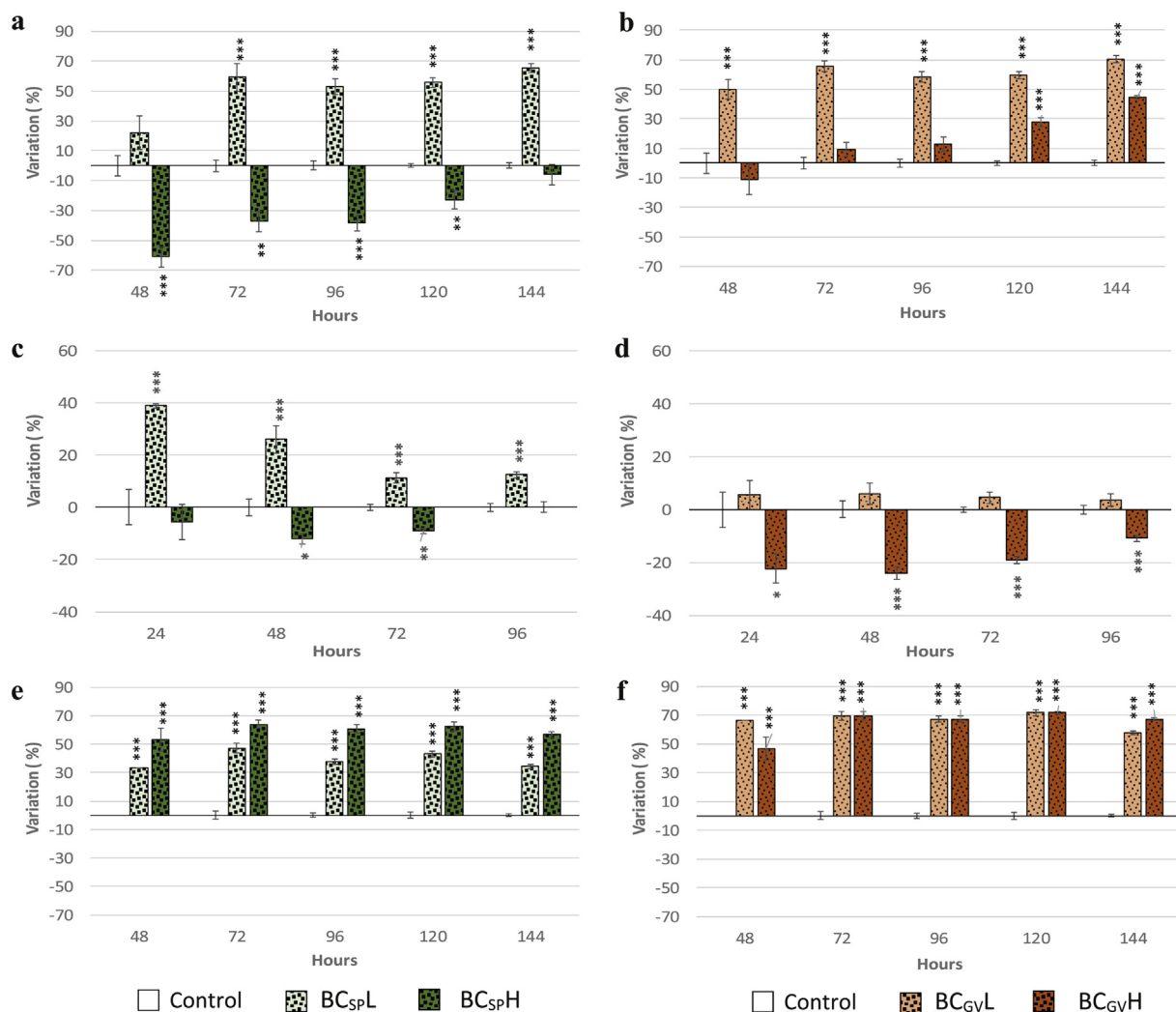


Fig. 1. Effects of BC samples at a concentration of 0.4% (w/v, L) and 2% (w/v, H) on variations (%) of the radial mycelial growth of *Trametes versicolor* (a and b), *Pleurotus ostreatus* (c and d) and *Pleurotus eryngii* (e and f), with respect to control (only error bar), as a function of time. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, according to LSD test. Standard error of the mean ($n = 5$) is also indicated.

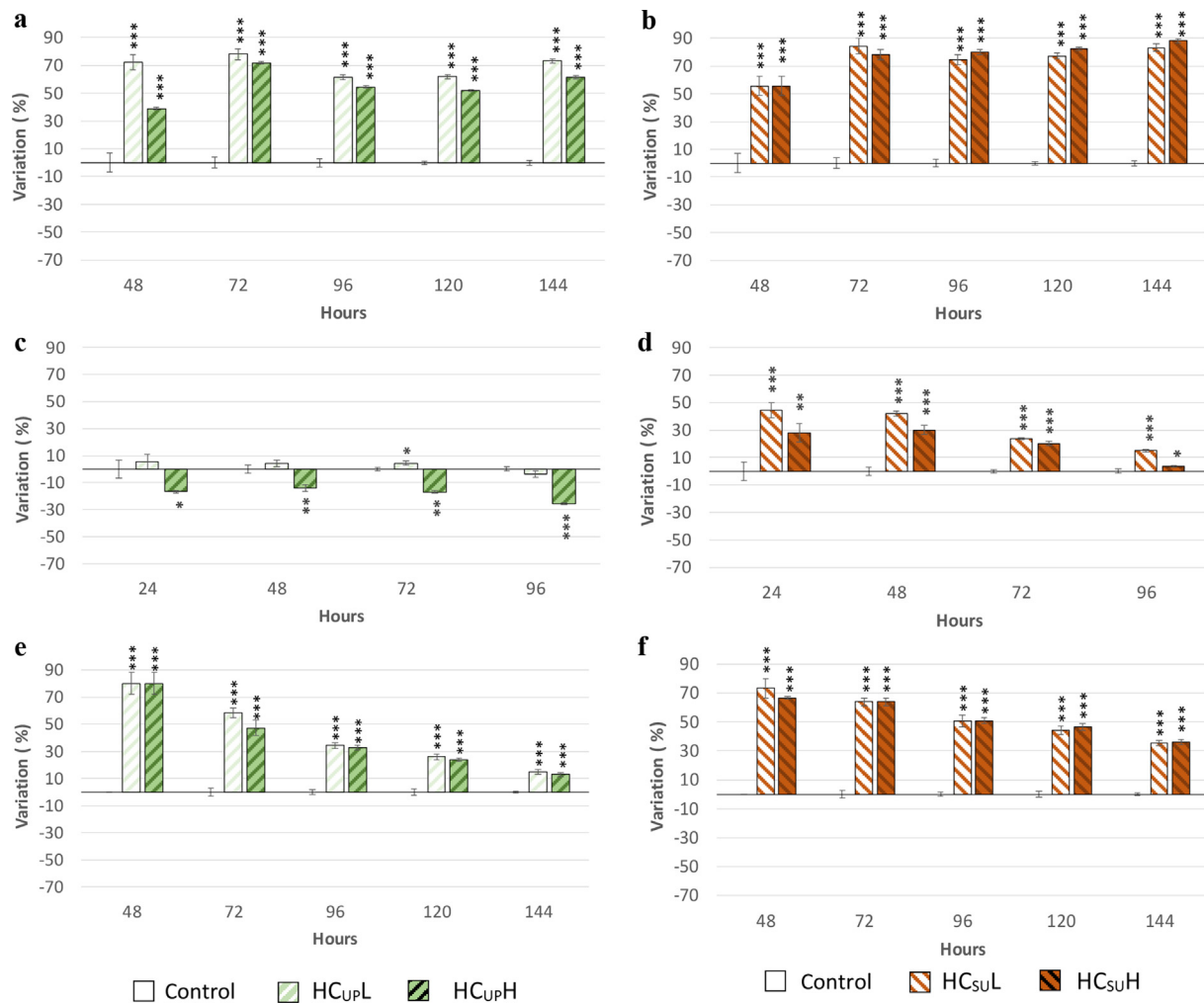


Fig. 2. Effects of HC samples at a concentration of 0.4% (w/v, L) and 2% (w/v, H) on variations (%) of the radial mycelial growth of *Trametes versicolor* (a and b), *Pleurotus ostreatus* (c and d) and *Pleurotus eryngii* (e and f) with respect to control (only error bar), as a function of time. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, according to LSD test. Standard error of the mean ($n = 5$) is also indicated.

are presented in Fig. 2. In comparison to BC, this amendment had a general better influence on all fungi. The presence of each HC at any dose produced a relevant stimulation on the growth of these fungi, with the only exception of HC_{UP}L and HC_{UP}H which caused, respectively, no effects or a weak inhibition on *P. ostreatus* (Fig. 2c). *T. versicolor* showed the highest increases of radial mycelial growth, which were in the ranges of 62%–78% and 39%–71%, respectively, in treatments with HC_{UP}L and HC_{UP}H (Fig. 2a). The same fungus was even more favourably influenced by HC_{SU}, with increases of mycelial growth in the range of 55%–88% for both doses (Fig. 2b).

The presence of HC_{UP}L stimulated the growth of *P. ostreatus* of about 4%, compared to control, only after 72h from inoculation (Fig. 2c). In contrast with the general trend, the presence of HC_{UP}H increasingly inhibited the growth of *P. ostreatus* from 17% at 24 h up to 26% at 96 h sampling times (Fig. 2c). Furthermore, *P. ostreatus* growth was significantly stimulated by HC_{SU} treatments at both concentrations, even if beneficial effects progressively decreased along the experimental period (Fig. 2d).

Similarly to what was observed for *T. versicolor*, *P. eryngii* was greatly stimulated by each dose of both HC samples with growth variations always significant ($P \leq 0.001$) compared to control (Figs. 2e and 2f). In particular, both doses of each HC produced the same results, with HC_{UP} and HC_{SU} variations ranging, respectively, from about 80% and 70% at the beginning of experiments to about 15% and 35% at the end of experiments (Figs. 2e and 2f).

3.2. Enzyme activity assays

Initial screening carried out on enzyme activity showed that the fungi examined in this study differed in enzyme machinery activated. In our experimental conditions, *T. versicolor* and *P. eryngii* produced laccase in both BC and HC treatments and control but did not produce MnP, whereas *P. ostreatus* produced measurable amounts of MnP only in treatment plates but no laccase.

3.2.1. Biochar

The effects of BC on the activity of selected enzymes released by *T. versicolor*, *P. ostreatus* and *P. eryngii* are presented in Table 1. The presence of each BC at the two doses always significantly induced a marked increase of enzyme activity of all fungal species.

In the case of *T. versicolor*, the highest laccase activity measured in all treatments was induced by BC_{SP}L treatment (75.8 IU L⁻¹) being 6.4 times higher than that measured in the control (Table 1). Even the higher dose of the same treatment (BC_{SP}H) significantly ($P \leq 0.01$) stimulated laccase activity. When BC_{GV} was added, both doses considerably increased laccase activity of *T. versicolor* compared to the control ($P \leq 0.01$), but the effects of the lower dose were less than those of the higher dose (Table 1).

Both BC types at both doses induced a relevant Mn-dependent peroxidase activity in *P. ostreatus*, whereas this activity was not measurable in the corresponding control. The highest MnP activity of this

Table 1

Effects of BC treatments at concentrations of 0.4% (w/v, L) and 2% (w/v, H) on enzyme activity of the three fungi. ** $P \leq 0.01$ and *** $P \leq 0.001$, according to LSD test. Standard error of the mean ($n = 3$) is also indicated.

Species – Enzyme	Enzyme Activity (IU L ⁻¹)				
	Control	BC _{SpL}	BC _{SpH}	BC _{GvL}	BC _{GvH}
<i>T. versicolor</i> - Laccase	11.9 ± 0.1	75.8 ± 5.0***	61.9 ± 6.7**	28.3 ± 2.6**	72.7 ± 15.1***
<i>P. ostreatus</i> - MnP	0.0 ± 0.0	30.4 ± 1.6***	64.8 ± 0.5***	23.8 ± 1.9***	52.8 ± 6.6***
<i>P. eryngii</i> - Laccase	42.6 ± 1.9	69.9 ± 4.3**	173.1 ± 20.8***	287.5 ± 36.7***	222.8 ± 17.5***

fungus was measured in BC_{SpH} treatment (64.8 IU L⁻¹). MnP activity was 23.8 IU L⁻¹ in BC_{GvL} treatment and more than twice higher in BC_{GvH} treatment (Table 1).

Similarly to what was already observed for the previous fungi, laccase activity of *P. eryngii* was always stimulated by the presence of BC in the fungal substrate (Table 1). The highest activity was measured in BC_{GvL} treatment (287.5 IU L⁻¹). Laccase activity of *P. eryngii* in BC_{SpH}, BC_{GvL} and BC_{GvH} treatments was much higher than that measured in the same treatments with *T. versicolor*, being about 3, 10 and 3 times higher, respectively (Table 1).

3.2.2. Hydrochar

The effects of HC on enzyme activity of the three fungi is presented in Table 2. The presence of each HC at both doses significantly increased enzyme activity of all fungi, with respect to control, with the only exception of HC_{SuL} on *P. ostreatus* that did not differ from control. Similarly to what was observed in BC treatments, the highest increases of enzyme activity were measured for *P. eryngii* (Table 2).

In the case of *T. versicolor*, the highest laccase activity was found in HC_{UpH} treatment (246.8 IU L⁻¹). Laccase activity was significantly stimulated also by the lower dose of this HC, being more than 4 times higher than that measured in the control (11.9 IU L⁻¹). Furthermore, enzyme activity of this fungus was much stimulated by both HC_{Su} treatments (Table 2).

Also for *P. ostreatus*, the highest enzyme activity was measured in HC_{UpH} treatment (83.7 IU L⁻¹). At the lower dose of the same BC (HC_{UpL}), MnP activity of *P. ostreatus* was only one-fourth of the activity measured in HC_{UpH} treatment (Table 2). Only the higher dose of HC_{Su} significantly stimulated MnP activity of the fungus when compared to control (Table 2).

The presence of each HC at both doses in the growth medium of *P. eryngii* significantly ($P \leq 0.001$) stimulated laccase activity compared to control. Once again, among all treatments, the highest activity was measured for HC_{UpH} (544.9 IU L⁻¹). The lower dose of the same sample (HC_{UpL}) increased laccase activity of the fungus only slightly less than the higher dose. Further, significant enhancements of laccase activity were recorded in both HC_{Su} treatments compared to control (Table 2).

Table 2

Effects of HC treatments at concentrations of 0.4% (w/v, L) and 2% (w/v, H) on enzyme activity of the three fungi. ** $P \leq 0.01$ and *** $P \leq 0.001$, according to LSD test. Standard error of the mean ($n = 3$) is also indicated.

Species - Enzyme	Enzymatic Activity (IU L ⁻¹)				
	Control	HC _{UpL}	HC _{UpH}	HC _{SuL}	HC _{SuH}
<i>T. versicolor</i> - Laccase	11.9 ± 0.1	50.9 ± 3.3***	246.8 ± 23.0***	208.9 ± 20.4***	116.0 ± 21.0**
<i>P. ostreatus</i> - MnP	0.0 ± 0.0	21.2 ± 1.5***	83.7 ± 3.5***	2.4 ± 2.4	67.6 ± 3.6***
<i>P. eryngii</i> - Laccase	42.6 ± 1.9	451.5 ± 27.9***	544.9 ± 17.8***	340.9 ± 23.0***	379.8 ± 13.9***

4. Discussion

4.1. Growth assays

The overall results achieved in the present study indicated that BC can exert beneficial effects on the growth of the fungal species tested. However, inhibitory effects on these fungi were also observed in some BC treatments at the higher concentration to an extent depending on BC origin, dose applied and fungal species. Compositional differences between the two BCs might have been responsible of the differentiated effects exerted on the same fungus. In this respect, the interpretation of the results of the present study may relies, at least partly, on the chemical properties of our BCs (and HCs), which are reported in a recent related article (Taskin et al., 2019). The elemental composition of the two BCs studied showed quite low H/C ratios suggesting the occurrence in these samples of highly condensed aromatic structure (Taskin et al., 2019). Moreover, gas chromatography/mass spectrometry analysis of these BCs evidenced the presence of aromatic hydrocarbons, including naphthalene, benzene, styrene, xylene and ethylbenzene (Taskin et al., 2019). In the treatments at the higher BC dose, these compounds might have exerted suppressing effects on fungi. Recently, Zhang et al. (2018) reported that the presence of recalcitrant aromatic compounds in BC is unfavorable for the fungal growth and the activity of ligninolytic enzymes. In some cases, inhibition induced by BC on mycelial growth appeared progressively diminish or disappear in the end of experiments, conceivably due to the activation of biochemical mechanisms of detoxification. In particular, BC treatments at the lower dose (0.4%, w/v) caused mostly growth stimulation on *T. versicolor* and *P. ostreatus*, while the higher dose of BC showed some inhibition. Differently, Ascough et al. (2010) found depressive effects of BC at a concentration at 0.5% (w/v) on the growth of *P. pulmonarius* and *Coriolus versicolor* (syn. *T. versicolor*). Zhang et al. (2018) reported that the growth of microorganisms, including fungi, had significant positive correlations with the aliphatic C but negative correlations with the aromatic C of different BCs. On the other hand, all BC treatments adopted showed a tremendous stimulation of *P. eryngii* growth. That suggests a species-dependent response of ligninolytic fungi to BC. That is consistent with the findings of Ascough et al. (2010) who, in a study carried out using BC produced at 300 °C and 400 °C from Scots pine, found dose- and species-dependent impact on fungi *P. pulmonarius* and *C. versicolor*. In the same study, the researchers observed that only *P. pulmonarius* responded differently to BC produced at different temperatures (Ascough et al., 2010). It is reasonable to assume that some components of BC released in the medium and absorbed by the fungus exerted regulatory effects on mycelial growth, some acting as stimulants, some as inhibitors and others in one way or another depending on the dose (Giller et al., 1998). For instance, heavy metals at high concentrations are generally toxic to ligninolytic fungi but some of them at low concentrations are also essential for growth and biological functions (Baldrian, 2003; Asif et al., 2017). Tolerance to high levels of heavy metals can vary significantly among fungal species and within the same species (Giller et al., 1998). In our study, the presence in BC of essential microelements such as Cu, Fe and Mn, might have promoted fungal growth. As a matter of facts, BC_{Sp} had much higher contents of Fe (about 4.3 mM) and, especially, Mn (2.5 mM) compared to BC_{Gv} (Taskin et al., 2019) and that might explain the absence of stimulation of *P. ostreatus* by the latter material. On the other hand, the presence in BCs of As, Pb and Cl, at levels conceivably higher than those tolerated by the fungus, might have caused inhibition. Asif et al. (2017) reported that even very low levels of Pb and Cl can cause inhibition of ligninolytic fungi. The growth inhibition caused by the higher BC dose (2%, w/v) observed in our study is in agreement with results of Gibson et al. (2016). These authors found that a BC produced at 450 °C from *Pinus ponderosa* at a dose of 5% (w/v) decreased respiratory rate of *T. versicolor* (Gibson et al., 2016). However, in some cases, BC inhibition at the higher dose measured in our study seemed successfully overcome by adaptation of the fungus to the growth conditions, possibly due to some changes in type

and quantity of enzymes produced or activation of biochemical mechanisms of detoxification. Other authors focused their studies on the impact of BC on mycorrhizal fungi. Testing a BC obtained at a temperature of 550 °C from spruce and pine wood pellets on the growth of *Rhizopagus irregularis*, Hammer et al. (2014) demonstrated that the fungus was capable to grow well on and into BC and to acquire phosphorus from it.

Due to the very recent production and use of HC, only little information can be found in the literature on the effects of this material on microorganisms, and almost none concerning its impact on soil-borne fungi. Álvarez et al. (2017) found that microbial biomass carbon and the activity of some enzymes could be influenced by the presence of HC, even if the final effects depended strongly on HC type. Due to the different production technology, HC can be quite different from BC as far as chemical and physical properties, including quantity and quality of aromatic components and mineral elements, are concerned. The lower temperatures adopted in HC production mitigate degradation reactions like decarboxylation, dehydration and aromatization that result in a C content significantly lower in this material than in BC. The two types of HC used in this work showed similar properties, except for pH and ash content which were lower for HC_{UP} (6.6 and 12%, respectively) than HC_{SU} (7.7 and 16%, respectively) (Taskin et al., 2019). It is reasonable to hypothesize that the lower alkaline nature and the lower degree of aromaticity of HC, compared to BC, make HC more acceptable by the tested fungi than BC. Both HCs studied here, especially HC_{SU}, showed levels of Cu, Fe and Zn higher than those found in BC samples (Taskin et al., 2019), and that might have promoted fungal growth. Anyway, HC also contains some toxic aromatic components that might explain the slight inhibition caused by HC_{UP} on *P. ostreatus*. Soil amendment with HC from different feedstocks was found to promote overall fungal biomass in soil (Steinbeiss et al., 2009) and, particularly, that of arbuscular mycorrhizal fungi (Rillig et al., 2010; George et al., 2012).

4.2. Enzyme activity assays

In addition to the important effects on the growth of the fungi examined, this study demonstrated that the presence of BC and HC in the substrate can also significantly stimulate enzyme activity of ligninolytic fungi. Between the two materials tested, HC demonstrated a general greater capacity to increase enzyme activity. That might be ascribed to the different composition of the two types of materials as it regards both the organic and the mineral fraction. Compared to the BC samples, the two HCs studied showed higher contents of easily degradable organic compounds and, with few exceptions, higher contents of all measured mineral elements, especially Cu, Fe and Zn (Taskin et al., 2019) which are essential elements for fungal growth and enzymes production and activity (Asif et al., 2017). In a recent work, Zhang et al. (2018) found positive correlations between ligninolytic enzyme activities and the aliphatic fraction of different chars, but also negative correlations with their aromatic fraction. These authors concluded that the increase in recalcitrant aromatic C in BCs could be unfavorable for the microbial growth and enzyme activity (Zhang et al., 2018). Anyway, there is scarce information in the literature on this matter, and only one study concerned the impact of BC on enzyme activity of *P. ostreatus* (García-Delgado et al., 2015). Therefore, the novelty of this work is to show that BC and HC greatly enhance the constitutive production of laccase by *T. versicolor* and *P. eryngii* and also activate the inducible production of MnP by *P. ostreatus*.

In our experimental conditions, we found that *P. ostreatus* released MnP system but not laccase, at least not enough laccase to be detectable. The marked increase of MnP activity observed in plates inoculated with *P. ostreatus* and the equally relevant increase of laccase activity in plates inoculated with the other two fungi is in accordance with what was observed by García-Delgado et al. (2015). In their study, the authors found that a BC obtained from pine wood chips at the temperature of 450 °C added at a concentration of 2.5% (w/v) stimulated the laccase activity

of *P. ostreatus*. Furthermore, a study of Placido et al. (2016) demonstrated that white-rot species, such as *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora*, *Postia placenta* and *Bjerkandera adusta*, were able to depolymerize BC using their laccase and MnP systems. The species-dependent response of fungi to BC, in terms of enzyme activity stimulation, is in agreement with the findings of Placido et al. (2016) who found that different white-rot fungi reacted differently to BC application in terms of enzyme activity levels and depolymerization rates. Soil amendment with carbon rich material (carbon nanotubes) was found to promote laccase activity of *T. versicolor* (Berry et al., 2014).

Unfortunately, no studies are present in the literature about the effects of HC on enzyme activity of ligninolytic fungi and a comparison between our results and those of other researchers is not possible. However, it is known that HC has fewer aromatic structures and a higher percentage of labile C fractions than BC (Cao et al., 2011; Gascó et al., 2018). HC samples used in this study were either from lignocellulosic material only (urban pruning residues) or from feedstock containing lignocellulosic material (solid urban wastes). In contrast to pyrolysis, hydrothermal carbonization process occurs at lower temperature and materials are not completely carbonized. Spectroscopic analyses of various HC samples (Liu et al., 2013), including the two HCs examined in this work (Taskin et al., 2019), showed the presence of aromatic groups of lignin, indicating that lignin from original feedstock was still present in HC after hydrothermal carbonization process. We can hypothesize that the lignin fraction present in HC, along with the cellulose and hemicellulose fractions, might have contributed to the generally higher stimulation of enzyme activity observed in HC, with respect to BC. Furthermore, the relevant increase of enzyme activity caused by HC addition in fungal substrate, compared to the control, is in agreement with the general stimulation of microbial activity observed by Kamman et al. (2012) in a soil incubated with HC from beet root chips and bark chips. Furthermore, mineral elements of HC and BC might have understandably affected at the same time both the growth of fungi and the production and activity of their enzymes, even if not at the same extent. A large number of studies addressing the influence of the quantity and type of metals on fungal enzymatic activity have been recently summarized by Asif et al. (2017). Baldrian (2003) reported an increase in the production and/or activity of fungal laccase in the presence of essential metals such as Cu, Fe, Mn and Zn. It is known that low concentrations of essential metals are necessary for the development of the ligninolytic enzyme system (Baldrian, 2003). In particular, Mn is directly involved in the catalytic cycle of Mn-dependent peroxidase (MnP) and Cu is the cofactor of the enzyme laccase. The positive effect of Cu addition on the production of laccase was observed in a large number of ligninolytic fungi (Baldrian, 2003). Stajic et al. (2013) reported that the enrichment of fungal growth medium with Zn caused an increase of laccase activity by *P. ostreatus*. Therefore, the higher activity of laccase by *P. eryngii* observed in treatments with HCs, compared to those with BCs, might be due, at least partly, to the overall higher contents of Zn, Cu and Fe of HC compared to BC (Taskin et al., 2019). The contents of Cu, Fe and Zn were, averagely for the two samples, 6, 16 and 5 times higher, respectively, in HCs than in BCs.

Finally, comparing the two HCs of this study, it appeared that HC_{SU} stimulated the enzyme activity slightly less, especially at the higher dose, than HC_{UP}. As the quantity and type of the organic fraction of HC_{UP} and HC_{SU} were similar (Taskin et al., 2019), we can assume that a significant role was played by the mineral components of HCs. The elemental composition of HCs showed Cu, Fe, Mn and Zn concentrations in HC_{SU} that were, respectively, 11, 3, 3 and 9 times higher than in HC_{UP}. It is reasonable to hypothesize that these metals, although essential, may have limited enzyme stimulation beyond certain levels. Moreover, HC_{SU} had contents of Pb and Ni, which are considered toxic nonessential heavy metals, about 6 and 3 times higher than HC_{UP} and that might have had a role in the results observed. Previous work reported that biochemical properties, including enzymatic activities, of HC were strongly dependent on HC type (Álvarez et al., 2017).

5. Conclusions

This study provided additional information about a subject not yet explored enough, that is the interaction between soil amendments, such as BC and HC, and soil-resident ligninolytic fungi. The overall results obtained demonstrated that the addition of these materials to the fungal medium generally favoured the growth and enzymatic activity of some ligninolytic fungi. However, aspects concerning feedstock, production conditions, dose applied, and the fungal species interacting with the material are very important for final results.

In the conditions of our study, the fungi examined appeared to accept HC better than BC demonstrating a better growth and a more relevant increase of enzyme activity. Finally, our results not only contribute to the comprehension of the biological processes occurring in soil following applications of BC and HC but also suggest a joint use of BC or HC and ligninolytic fungi in remediation processes, in order to combine the adsorptive capacities of the materials with the degradative actions of fungi.

Declarations

Author contribution statement

Elisabetta Loffredo, Eren Taskin: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Maria Teresa Brana: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Claudio Altomare: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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