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Combination of biochar amendment and mycoremediation for polycyclic aromatic hydrocarbons immobilization and biodegradation in creosote-contaminated soil

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Abbreviations:

Polycyclic Aromatic Hydrocarbons (PAH), fluorene (Flu); phenanthrene, (Phe), anthracene (Ant), fluoranthene (Fla), pyrene (Py), benzo[a]anthracene (BaA), chrysene (Chr), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylene (BghiP), indeno[c,d]pyrene (IcdP), High Molecular Weight (HMW), Non amended Soil (S), soil amended with biochar (B), soil amended with wheat straw (WS), mycoremediation treatment (P), combination of B and P treatment (BP), manganese dependent peroxidase (MnP), Lignin-peroxidase (LiP), unit of enzyme activity (IU), most probable number (MPN), hydroxypropyl-β-cyclodextrin (HPCD), Fluorescin diacetate (FDA), Ionization Potential (IP), day (d).

Abstract

Soils impregnated with creosote contain high concentrations of polycyclic aromatic hydrocarbons (PAH). To bioremediate these soils and avoid PAH spread, different bioremediation strategies were tested, based on natural attenuation, biochar application, wheat straw biostimulation, *Pleurotus ostreatus* mycoremediation, and the novel sequential application of biochar for 21 days and *P. ostreatus* 21 days more. Soil was sampled after 21 and 42 days after the remediation application. The efficiency and effectiveness of each remediation treatment were assessed according to PAH degradation and immobilization, fungal and bacterial development, soil eco-toxicity and legal considerations. Natural attenuation and biochar treatments did not achieve adequate PAH removal and soil eco-toxicity reduction. Biostimulation showed the highest bacterial development but low PAH degradation rate. Mycoremediation achieved the best PAH degradation rate and the lowest bioavailable fraction and soil eco-toxicity. This bioremediation strategy achieved PAH concentrations below Spanish legislation for contaminated soils (RD 9/2005). Sequential application of biochar and *P. ostreatus* was the second treatment most effective for PAH biodegradation and immobilization. However, the activity of *P.ostreatus* was increased by previous biochar application and PAH degradation efficiency was increased. Therefore the combined strategy for PAH degradation have high potential to increase remediation efficiency.

Keywords: biodegradation, bioavailability, *Pleurotus ostreatus*, ligninolytic enzymes, biochar

1. Introduction

Creosote is a chemical formulation obtained from coal-tar distillation used in wood preservation. Its composition is about 85% polycyclic aromatic hydrocarbons (PAH),

compounds with toxic, carcinogenic and mutagenic properties. In fact, creosote was indexed as *probably carcinogenic to humans* (Group 2A) by the International Agency for Research on Cancer [1]. The industrial process of wood preservation involved impregnating wood with creosote in pressurized tanks to encourage creosote penetration. Then, the creosote-impregnated wood is dried in a field storage area prior to use. In this step, the creosote evaporates into the atmosphere, impregnates the soil and can leach into groundwater [2,3] The consequences of the process are soil, water and air pollution of the area. Therefore, the monitoring and remediation of soil creosote wood treatment plants are necessary to prevent contaminants spread.

The most studied biological strategies to remediate creosote polluted soil are biostimulation and biomagnification [4]. Both strategies use organic amendments or additives to improve nutrient status or physico-chemical conditions with the aim of improving microbiological development and the degradation of contaminants. However, these materials are able to modify the contaminants bioavailability because they can act as contaminant sorbents or mobilizer agents [5]. Beesley et al. [6] described a reduction in PAH bioavailability due to the application of biochar and green waste compost to soil. Wu et al. [5] discussed PAH sorption/desorption observed during soil incubation with mature compost and the implications for PAH biodegradation because contaminants desorption is a critical factor in bioremediation.

Soil bioremediation with bacteria involves the transfer of PAH from soil to bacterial cells, hence the low PAH bioavailability; mainly high molecular weight (HMW) PAH results in a low mass-transfer rate from soil to bacterial cells. Thus, the degradation rate of HMW-PAH is slow and correlates with the bioavailable fraction [4,7]. Therefore, one option for optimizing PAH biodegradation with bacteria involves increasing

contaminant bioavailability. However contaminants desorption can result in environmental risk because of the possibility of contaminants spreading.

To solve this problem, ligninolytic fungi have been suggested as an adequate option to biodegrade PAH with low bioavailability [8]. Ligninolytic fungi segregate extracellular ligninolytic enzymes (peroxidases and/or laccase) that have low substrate specificity, are able to diffuse into the soil matrix and potentially oxidize PAH with low bioavailability. Covino et al. [9] showed the ability of *Dichomitus squalens*, *Coprinus comatus* and *Pleurotus ostreatus* to degrade certain PAH beyond their respective bioavailable amount in soil. Therefore the bioremediation of PAH contaminant soils based on PAH sorption and biodegradation with ligninolytic fungi appears as an interesting strategy to minimize risks of contaminant leaching.

Consequently, the aims of this study were to assess the PAH immobilization and degradation effectiveness and efficiency of four bioremediation strategies (biochar amendment, biostimulation with wheat straw, mycoremediation with *P. ostreatus* and sequential application of biochar and *P. ostreatus*) for a creosote-contaminated soil with respect to natural attenuation and to determine the reduction of soil ecotoxicity according to total microbial activity and seed germination test.

1. Materials and methods

1.1. Materials

Polluted soil was collected from a creosote wood treatment plant located in Castejón (Spain). Soil samples were collected from wood stock area. Then, soil samples were pooled, homogenized, air-dried at room temperature and, finally, passed through a 2 mm-sieve. Main soil properties were: pH 8.38±0.03 and electric conductivity 0.324±0.014 dS m^{-1} in aqueous extract 1:5 (w:v); $CaCO_3$ 36±1%; organic matter

1.11±0.04%. Texture composition was as follows: sand, 19%; silt, 33%; clay, 48%. According to the US textural classification, it was a clay soil with a water holding capacity of 37%. Thirteen of the 16 US EPA PAH were present in the polluted soil; the PAH concentrations are shown in Table 1.

The selection of biochar amendment was based on soil PAH immobilization properties and biodegradation enhancement [10].Three biochars were tested in a preliminary study of *P. ostreatus* growth: maize, willow and pine. The last was the one showing fungal development without any additional carbon source and was therefore selected for the bioremediation assay.

Organic amendments used were: i) pine woodchip biochar that was produced at 450ºC and previously characterized and used by [11] for metals immobilization, and ii) wheat straw, good lignocellulosic substrate as carrier for *P. ostreatus* inocula [9] and adequate material for soil fungi and bacteria biostimulation [12].

1.2. Remediation treatments

Remediation experiments were carried out in 1-l glass reactors and the contaminated soil (40 g dry mass) was used without sterilization. The organic amendments were sterilized in autoclave (121 °C, 30 min) before application in the soil to ensure that PAH removal was done by indigenous soil microbiota or the inoculated fungi (*P. ostreatus*).

Soil moisture content in all treatments was adjusted to 70% of water holding capacity prior to the beginning of incubation. The treatments were designed in order to assess the following remediation processes:

- Non amended soil (S treatment): control treatment that simulated natural attenuation.

- Biochar amendment (B treatment): the polluted soil was amended with sterilized pine biochar (2.5%). This treatment was designed to promote PAH degradation and immobilization simultaneously, in order to minimize PAH leachate risks.

- Biostimulation of soil microbiology (WS treatment): The polluted soil was amended with sterilized wheat straw (25%). This approach was intended to assess the stimulatory effect of sterilized wheat straw on soil-resident microbiota.

- Mycoremediation (P treatment): sterilized wheat straw was inoculated with 3 agar plugs (diameter, 1 cm) from 14-d-old *P. ostreatus* cultures on malt extract agar and incubated for 14d at 28 °C. Then, the colonized matrix was mixed with the contaminated soil (1:4, wheat straw: soil). This bioaugmentation approach aimed at determining the efficiency of mycoremediation.

- Sequential biochar amendment and mycoremediation (BP treatment): The objectives of the treatment were: firstly, immobilize PAH and finally biodegrade PAH by *P. ostreatus*. This remediation treatment was constructed as biochar treatment for 21d. Then *P. ostreatus* was inoculated, as described above, and incubated during 21d more.

Each treatment was carried out in triplicate and incubated at 28 °C for 21 and 42d under static conditions in the dark.

1.3. Ergosterol and ligninolytic enzyme analysis

Total soil ergosterol content was extracted as described by [9]. Ergosterol was analysed by HPLC (Waters 2695 Separation Module, Waters Milford, MA) equipped with a Phenomenex Luna C18 column (250mm×4.60mm; particle size 5 µm; pore size 100 A) equilibrated with methanol:water (95:5) at a flow rate of 1mL min^{-1}. Sample injection volume was 20 µL. The elution profile was monitored at 282 nm.

Ligninolytic enzymes were extracted from soil samples (3 g) at 5 °C for 1 h using the buffered solution described by [13]. Then, the aqueous suspension was centrifuged (6000 g, 30 min) and the supernatant was assayed for ligninolytic activities.
Laccase activity was spectrophotometrically determined by following the oxidation of 0.2 mM 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid in 100 mM sodium acetate buffer (pH 4.5) at 420 nm (ε= 36000 M^{-1} cm^{-1}). Manganese dependent peroxidase (MnP) activity was assayed by the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate buffer (pH 4.5), in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{3+}, form a complex with malonate, which absorbs at 270 nm (ε= 11590 M^{-1} cm^{-1}) [14]. Lignin-peroxidase (LiP) was determined by oxidation of veratryl alcohol in 100 mM sodium tartrate buffer (pH 3.0), in the presence of 0.4 mM H_2O_2. Veratryl aldehyde was determined at 310 nm (ε= 9300 M^{-1} cm^{-1}). One unit of enzyme activity (IU) is defined as the amount of enzyme producing 1µmol of product per minute under assay conditions.

1.4. Estimation of heterotrophic and PAH-degrading bacteria

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method in 96-well microtitre plates, with eight replicate wells per dilution according to [15]. To avoid fungal development, cycloheximide at 100 mg l^{-1} final

concentration was added to both growth media. The MPN calculation was carried out with the US EPA MPN Calculator v1.1 software.

1.5. Total and bioavailable PAH analysis

Total extraction and analysis of PAH were performed according to [16]. The PAH bioavailable fraction for soil microorganisms was determined by hydroxypropyl-β-cyclodextrin (HPCD) extraction [17]. PAH detected and quantified were: fluorene (Flu); phenanthrene, (Phe), anthracene (Ant), fluoranthene (Fla), pyrene (Py), benzo[a]anthracene (BaA), chrysene (Chr), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylene (BghiP) and indeno[c,d]pyrene (IcdP).

1.6. Estimation of soil ecotoxicity

Soil total microbial activity as soil health parameter, was determined by hydrolysis of fluorescein diacetate (FDA) [18].

Soil germinability test with 25 *Lactuca sativa* seeds were conducted for 3d in darkness at 28ºC in 90-mm Petri dishes containing Whatman nº 42 filters soaked with 2.0 mL of acetone:hexane extracts obtained during PAH total extraction from original soil and remediation strategies at 42d per triplicate. After addition of PAH extracts, the filters were held at room temperature for 12h to allow solvent evaporation. Then, 2.0 mL of distilled water were added on a daily basis. Germinability tests conducted in the presence of distilled water were also run in parallel and served as the control. Germinated seeds and seeds with cotyledons develop were counted. Percentage of germinability was calculated from the following equation:

$$germinability\ (\%) = \frac{G}{G_c}\ .100$$

where G is the number of germinated seeds in the presence of PAH extract and Gc the same parameter in the absence of the PAH extract.

1.7. Statistical analysis

All statistical tests were carried out using the IBM SPSS Statistics v21 software package. One-way analysis of variance was carried out after previously performing a Levene variance homogeneity test. To compare the differences between treatments, the Duncan or Games–Howell *post hoc* test (according to variance homogeneity) at $p<0.05$ was used.

2. Results

2.1. Assessment of fungal development and ligninolytic activity

Fungal development was evaluated by ergosterol soil content (Fig. 1A). No ergosterol was detected in S and B treatments. WS treatment was able to develop indigenous fungi, mainly during the first 21d. Then the ergosterol concentration decreased drastically at 42d. *P. ostreatus* was able to colonize the polluted soil with wheat straw as carrier in both P and BP mycoremediation treatments. 21d after *P. ostreatus* inoculation in soil, P and BP treatment presented similar ergosterol concentration. However, 42d after *P. ostreatus* inoculation, P treatment achieved higher ergosterol content and therefore higher soil colonization.

With respect to ligninolytic activity, laccase, MnP and LiP were detected during the assay and used as fungal activity parameters (Fig 1B, C and D). No ligninolytic activity was detected in S treatment. Biochar promoted LiP activity and MnP at low levels. Indigenous fungi developing in WS treatment showed the highest LiP activity at 21d but LiP was not detected at 42d; MnP, on the other hand, showed low activity but constant during the assay. The highest laccase and MnP activities were obtained in the mycoremediation treatments (P and BP) and can be attributed to *P. ostreatus* activity.

These activities were higher at 42d after *P. ostreatus* inoculation except in BP treatment where laccase activity at 21d after *P. ostreatus* inoculation was similar to P treatment at 42d. Therefore, prior biochar amendment of the soil stimulated laccase activity for *P. ostreatus*.

2.2. Bacterial population

Densities of total heterotrophic and PAH-degrading bacteria are shown in Fig. 2. S treatment developed low total and PAH-degrading bacteria population at 21d. Total heterotrophic population was stable between 21 and 42d but PAH-degrading bacteria density decreased below detection limit from 21 to 42d. The method proposed by [15] to determine PAH-degrading bacteria population, is based on PAH as sole source of carbon. Therefore the bacteria present in the plates are PAH-degrading bacteria. However the method does not assure that other bacteria present in soil are not able to degrade PAH in presence of other carbon source. The treatment most effective to induce bacterial growth was WS. This treatment achieved the highest total and PAH-degrading bacterial populations. The biochar did not induce bacterial development and no significant differences for total heterotrophic bacteria were found between S and B treatments. No PAH-degrading population was detected in B treatment at 21 or 42d. However the inoculation of *P. ostreatus* in BP treatment induced a slight development of PAH-degrading bacteria at 42d and similar total heterotrophic bacteria density as WS at 42d. Treatment P got high total and PAH-degrading bacteria population at 21d but at 42d both bacterial counts were similar to S and B.

2.3. PAH removal

The PAH degradation rate for each treatment is shown in Table 1. The most degradable PAH were those with 3-rings (Flu, Phe and Ant), especially for S, B and WS treatments.

For P and BP treatments, both with *P .ostreatus*, no significant differences were found between degradation rates of PAH with 3 or 4-rings.

S and B treatments were the less effective remediation treatments. B treatment did not present a PAH degradation rate significantly higher than S at 42d, except for DBahA. However, at 21 d, the degradation rate of BghiP and Σ5,6-rings was higher than S, so B was able to increase the degradation speed of highly condensed PAH. WS treatment was more efficient than S and B with respect to Σ4-rings, Σ5,6-rings and Σ13PAH and some individual PAH such as Fla, Py and IcdP at 42d.

Remediation treatments that involved *P. ostreatus*, (P and BP) achieved the best biodegradation rates for total (73 and 58%, respectively) and individual PAH after 42d of incubation. P treatment got the highest degradation rates at 42d for all PAH. BP treatment was applied sequentially, firstly 21d of incubation with biochar and then 21d of incubation with *P. ostreatus*. The result was higher degradation of many PAH and all groups with respect to B treatment at 42d. The comparison of *P. ostreatus* action at 21d in P treatment and 42d in BP treatment showed that BP treatment achieved higher degradation rates for Phe, Ant, Fla, Py, Σ3-rings, Σ4-rings and Σ13PAH than P treatment. Thus, prior biochar application promoted PAH degradation by *P. ostreatus*.

2.4. Bioavailable PAH fraction

PAH bioavailability could be clustered by number of rings. The PAH bioavailability decreased when the number of rings increased, and consequently PAH with 5,6-rings were the less bioavailable compounds (Table 2). Changes of PAH bioavailability at 21d of incubation were slight. S, B or WS treatments did not show significant differences with respect to initial soil, except for IcdP in B and WS treatments, which decreased. P treatment decreased the bioavailability of BaP (60%) and IcdP (100%) at 21d. At 42d, S and B treatments did not immobilize PAH with respect to initial soil or 21d of

incubation. WS, P and BP treatments got lower PAH bioavailability than S and B treatments or initial soil. P treatment was the most effective for individual, groups or total PAH. The great bioavailability reduction of Σ5,6-rings from 24% at 21d to 2% at 42d of incubation is highlighted. The other mycoremediation treatment (BP), showed a similar trend. BP was able to reduce the bioavailability, with respect to S, B and WS treatments, of highly condensed PAH (4-rings and 5,6-rings) and Σ13PAH.

The PAH bioavailability evolution between 21 and 42d of incubation were different for each treatment. S and B treatments did not show significant differences. WS and P and BP treatments achieved a significant reduction of PAH bioavailability, mainly PAH with 4-rings and 5,6-rings. However, P and BP treatments achieved the lowest bioavailable rates. P treatment produced a strong reduction of 51and 92% of PAH with 4-rings and 5,6-rings respectively from 21 to 42d of incubation. The reduction was lower for BP treatment (61 and 46% respectively).

The organic amendments showed variable action in PAH bioavailability, pine biochar was not effective as PAH immobilizer and wheat straw was not effective as PAH sorbent in the short run (21d) but was adequate to reduce PAH bioavailability at 42d for HMW-PAH.

2.5. Soil Ecotoxicity

Figure 3 shows the FDA hydrolysis and germinability test for initial soil and remediation treatments after 42d. No FDA hydrolysis activity was detected in initial soil and B treatment achieving poor PAH degradation rates (17 and 14%). For WS, P and BP treatments, values of FDA hydrolysis presented the same trend as PAH degradation rates.

Results of germinability test with *L. sativa* (Fig. 3B) showed improvement of soil quality with WS, P and BP, having regard for total germination and seeds with

developed cotyledons. All remediation treatments other than natural attenuation (S) achieved higher germinability than initial soil. Thus, the natural attenuation process was not able to reduce soil phytotoxicity. Only P treatment showed a higher germination rate (78%) than S treatment. The rate of seed able to develop cotyledons was significant higher for WS, P and BP treatment than S and B treatments at the end of the incubation.

3. Discussion

This article reports the effectiveness and efficiency of five soil remediation strategies for PAH removal and/or immobilization. The initial Σ13PAH soil concentration was 1212 mg kg^{-1} and according to Spanish legislation [19], the soil was polluted by Fla, Py, BaA and BaP for industrial use.

Non amended soil (S treatment) achieved 17% of total PAH degradation after 42d of incubation, the final concentration of Σ13PAH was 1003±2 mg kg^{-1}. The bioavailable fraction was not modified over 42d. So the remediation efficiency of this treatment was very low and the results insufficient, as expected for natural attenuation.

The addition of biochar (B treatment) did not increase the effectiveness of the remediation at 42d but increased the degradation rate of PAH with 5,6-rings at 21d. The result contrasts with similar works where the application of biochar achieved significant PAH degradation [6,20]. The poor PAH degradation of this amendment was due to the low bacterial and fungal development (Fig 1 and 2) because the high C/N ratio (233, [11]) and most carbon in biochar has an aromatic structure with low biodegradability [20]. Consequently biochar achieved a low biostimulating effect. B treatment did not succeed for PAH immobilization. Only IcdP showed less bioavailability after 42d of incubation. Previous works on PAH immobilization with biochar [6,21] showed bioavailability reduction but biochar doses were higher than 10%. The biochar dose of

the present work was 2.5%, equivalent to a field application rate of approximately 110 t ha^{-1} This represents a high dose of organic amendment and a higher amount of biochar for field application did not appear feasible. Another factor that could explain the low PAH sorption capacity of the pine biochar was the low temperature (450ºC) of production. The higher the pyrolysis temperatures used, the higher the micropore content and the higher the organic contaminants sorption capacity[20].

Wheat straw was chosen for soil biostimulation because bioaugmentation treatments with *P. ostreatus* (P and BP) were carried out with the same organic material as fungal carrier. The low organic matter stability of this material can explain the low total PAH degradation (23%) at the end of the assay [22,23]. However, WS treatment was able to develop fungi and the highest total heterotrophic and PAH-degrading bacteria population at 21 and 42d. PAH degradation was more intense the first 21d because of the greater fungal development (ergosterol content) and ligninolytic activity, mainly LiP (Fig 1), and the higher bacterial population (Fig. 2). The PAH leachate risk was reduced with wheat straw application to soil because of the decrease in the bioavailable fraction of HMW-PAH with respect to S. The significant reduction in the bioavailable fraction from 21d to 42d partly explained the low degradation rate too. Despite the insufficient PAH degradation efficiency of WS treatment, soil biological stimulation with wheat straw achieved higher total and 4-rings PAH degradation, PAH immobilization and better biological parameters than S and B treatments. Hence WS treatment was more effective and efficient than S and B as bioremediation strategy.

The results offer clear evidence that mycoremediation with *P. ostreatus* was more efficient than biostimulation treatments (Table 1). The PAH degradation mechanism cannot be attributed only to ligninolytic activity because the activity of ligninolytic enzymes in P treatment was higher at 42 d (Fig. 1) but PAH degradation was more

intense during the first 21d. According to [24], the action of *P. ostreatus* responded to two possible mechanisms. Firstly, segregation of ligninolytic enzymes with proven capacity to degrade PAH. Secondly, in the growth phase of fungi and in the absence of necessary extracellular enzymes and/or co-substrates, the degradation of PAH could take place preferentially by aromatic compound uptake. In this regard, *Lentinus tigrinus* can degrade PAH via the intracellular cytochrome P-450/epoxide hydrolase complex [25]. So, it appears likely that PAH uptake and ligninolytic enzyme activity showed a synergistic effect during fungi colonization of the creosote contaminated soil.

The ionization potential (IP) of PAH ($7.12 \leq IP \leq 8.03$ eV) [9] and ligninolytic enzymes (laccase ≤ 7.45 eV, MnP ≤ 8.19eV, and LiP ≤ 7.55 eV) appears as an important factor in a one-electron oxidation of PAH [24]. BaP and DBahA are compounds with low IP (7.12 and 7.38 eV) [9] and achieved high degradation rates for P and BP treatments at 42d, probably because of combination effects of laccase that showed very high activity but low IP and MnP that showed low activity but high IP. Contaminants immobilization was not the main objective of P treatment but it was the most effective in this respect at 42d. The minimization of PAH bioavailability was higher in P than WS, although the organic matter applied was wheat straw at the same doses for both treatments. This may imply that the higher PAH degradation with P treatment was the main mechanism to decrease bioavailability. Therefore P treatment degraded the most bioavailable PAH fraction and the PAH degradation by *P. ostreatus* uptake could be an appreciable mechanism.

The sequential strategy of biochar and mycoremediation (BP treatment) was not the most effective strategy but showed an interesting behaviour with respect to fungal development, ligninolytic activity and PAH degradation. Ergosterol content of BP at 42d (21d after *P. ostreatus* inoculation) showed similar fungi colonization and MnP

activity than P treatment at 21d. And laccase showed more activity than P treatment at 21d and no significant differences at 42d, in the highest activity found in the assay (Fig 1). These results mean that amended soil with biochar prior to *P. ostreatus* application induced laccase segregation by the fungus. However, the increase in laccase activity did not mean a significant increase in PAH degradation for PAH with IP ≤ 7.45 (Ant, BaP, DBahA and BghiP) and degradation was more intense for Phe, Fla and Py (IP ≥ 7.45) probably because of interaction with the degradation of PAH by fungi uptake during the growth phase via the intracellular cytochrome P-450/epoxide hydrolase complex that is independent of IP. The results of the BP treatment in the *P. ostreatus* phase (from 21 to 42d) were a degradation increase of Phe, Ant, Fla, Py, Σ3-rings, Σ 4-rings and Σ13PAH with respect to P treatment at 21d and a degradation rate decrease of BghiP and IcdP. So prior soil amendment with biochar increased the efficiency of *P. ostreatus* in PAH degradation. With respect to B treatment, the PAH degradation of BP treatment was much more extensive in the last 21d for all PAH groups and Σ13PAH. Thus, the application of *P. ostreatus* contributed to increase the efficiency of the bioremediation process. The effect on the bioavailable fraction was similar to P treatment and the decrease in bioavailability fraction appeared more related to biodegradation efficiency than PAH sorption capacity of biochar and wheat straw.

As for legal considerations, only P treatment achieved PAH concentrations lower than the maximum allowed by Spanish legislation for industrial use (100 mg kg^{-1} for Fla and Py, 20 mg kg^{-1} for BaA and 2 mg kg^{-1} for BaP) [19]. S and B treatments were not able to decrease the concentration of Fla, Py, BaA and BaP below legal limits and therefore were not effective for soil bioremediation. BP treatment achieved concentrations below 100 mg kg^{-1} for Fla and Py but was not able to decrease the BaA and BaP below 20 and 2 mg kg^{-1} respectively in the remediation period (42d). It would probably need more

time to reduce the PAH concentrations below the permitted threshold. In this respect, P treatment was the most effective and efficient to bioremediate PAH-polluted soil and the soil can be declared decontaminated according to Spanish legislation.

Eco-toxicological tests (FDA hydrolysis and germinability, Fig. 3) confirmed the higher efficiency of biostimulation (WS) and mycoremediation (P and BP) than natural attenuation (S) or biochar (B) strategies for soil bioremediation. The potential of mycoaugmentation strategies was demonstrated by the higher microbial activity (Fig 3A) and plant development (Fig. 3B) of P and BP treatments. The development of *L. sativa* cotyledons showed better indications of PAH degradation than the simple germination test.

4. Conclusions

Bioremediation strategies for creosote-contaminated soil involving mycoremediation with *P. ostreatus* are more efficient and effective than non-amended soil or biochar and wheat straw application. The mycoremediation strategy (P treatment) achieves the best PAH biodegradation rate and the lowest bioavailable fraction and soil eco-toxicity. In addition, P treatment was able to reduce PAH concentrations to below Spanish limits for contaminated soils. Sequential application of biochar and *P. ostreatus* (BP treatment) increases fungal activity and PAH degradation capacity. In consequence, combined strategies for bioremediation appear to have high potential to increase remediation efficiency.

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Figure Captions

Figure 1: Fungal colonization and activity expressed as: A) concentration of ergosterol (mg kg^{-1}), B) laccase activity (IU kg^{-1}), C) Mn-Peroxidase activity (IU kg^{-1}) and D) lignin-peroxidase (IU kg^{-1}) for each remediation strategy: non amended soil (S), biochar amended soil (B), biostimulation (WS), mycoremediation (P) and sequential B and P strategy (BP). nd parameter not detected.

Figure 2: Heterotrophic (A) and PAH-degrading (B) populations in soil remediation treatments over the 21 and 42d of incubation. Same lowercase and uppercase letters indicate lack of statistically significant difference (p>0.05) between remediation treatments and sampling time, respectively. S: non amendment soil, B: biochar amended soil, WS: biostimulation, P: mycoremediation and BP: sequential B and P strategy.

Figure 3: Ecotoxicological test: microbial total activity (A) and percentage of *L. sativa* seed germination and developed cotyledons for initial soil (S d0), non amended soil (S d42), biochar amended soil (B), biostimulation (WS), mycoremediation (P) and sequential B and P strategy (BP) at the end of the assay (42d). Same letters indicate lack of statistically significant difference (p>0.05) between treatments. nd parameter not detected.

Table 1: Initial PAH subsoil concentration (mean ± standard deviation) and PAH degradation rate at 21 and 42 days of incubation in non amended subsoil (S), biochart treatment (B), bioaugmentation treatment with wheat straw (WS), mycoremediation treatment with *P. ostreatus* (P) and sequential biochar amendment plus mycoremediation (BP). Results are expressed as mean of three independent assays.

	Subsoil [PAH] mg Kg^{-1}	PAH degradation rate (%)								
		S		B		WS		P		BP
		21d	42d	21d	42d	21d	42d	21d	42d	42d
Flu	21.5±2.3	31^{ab}	40^{ab}	18^{a}	34^{a}	44^{b}	44^{b}	70^{c}	79^{c*}	72^{*c}
Phe	339±28	19^{a}	41^{b}	11^{a}	25^{a}	30^{ab}	32^{ab}	43^{b}	76^{d*}	$62^{c*\dagger}$
Ant	30.8±2.4	14^{a}	30^{a*}	15^{a}	24^{a}	46^{ab}	36^{a}	67^{b}	72^{b}	$79^{b*\dagger}$
Fla	341.8±2.3	4^{a}	6^{a}	8^{a}	7^{a}	20^{b}	19^{b}	36^{c}	71^{d*}	$58^{c*\dagger}$
Py	298±18	5^{a}	7^{a}	10^{a}	10^{a}	19^{b}	20^{b}	46^{c}	80^{d*}	$64^{c*\dagger}$
BaA	84.5±16	4^{a}	6^{a}	10^{a}	7^{a}	13^{a}	14^{a}	31^{b}	68^{c*}	40^{b*}
Chr	56.0±4.5	4^{a}	6^{a}	10^{a}	7^{a}	11^{a}	11^{a}	26^{b}	52^{c*}	31^{b*}
BbF	18.2±3.8	4^{a}	6^{a}	9^{ab}	8^{a}	9^{ab}	11^{a}	16^{b}	35^{c*}	19^{b}
BkF	6.28±0.95	4^{a}	6^{a}	10^{ab}	7^{a}	8^{ab}	11^{a}	16^{b}	26^{b}	8^{a}
BaP	7.56±0.38	11^{a}	13^{a}	17^{a}	15^{a}	15^{a}	20^{a}	59^{b}	81^{c*}	55^{b*}
DBahA	1.28±0.63	13^{a}	0^{a}	59^{b}	58^{b}	41^{ab}	52^{b}	75^{b}	94^{c}	89^{c*}
BghiP	3.19±0.28	0^{a}	11^{a}	22^{b}	21^{a}	24^{b}	25^{ab}	40^{b}	40^{b}	$18^{a\dagger}$
IcdP	3.39±0.22	0^{a}	0^{a}	10^{ab}	2^{a}	3^{a}	20^{b*}	27^{b}	30^{c}	$7^{a\dagger}$
Σ3-rings	391±32	19^{ab}	40^{b}	12^{a}	25^{a}	32^{bc}	33^{ab}	46^{c}	76^{c*}	$63^{b*\dagger}$
Σ4-rings	780±42	5^{a}	6^{a}	9^{a}	8^{a}	18^{b}	19^{b}	39^{c}	73^{d*}	$57^{c*\dagger}$
Σ5,6-rings	39.9± 1.8	4^{a}	6^{a}	14^{b}	11^{ab}	11^{b}	16^{b}	29^{c}	44^{d*}	25^{c*}
Σ13 PAH	1212±76	9^{a}	17^{a*}	10^{a}	14^{a}	22^{b}	23^{b}	41^{c}	73^{d*}	$58^{d*\dagger}$

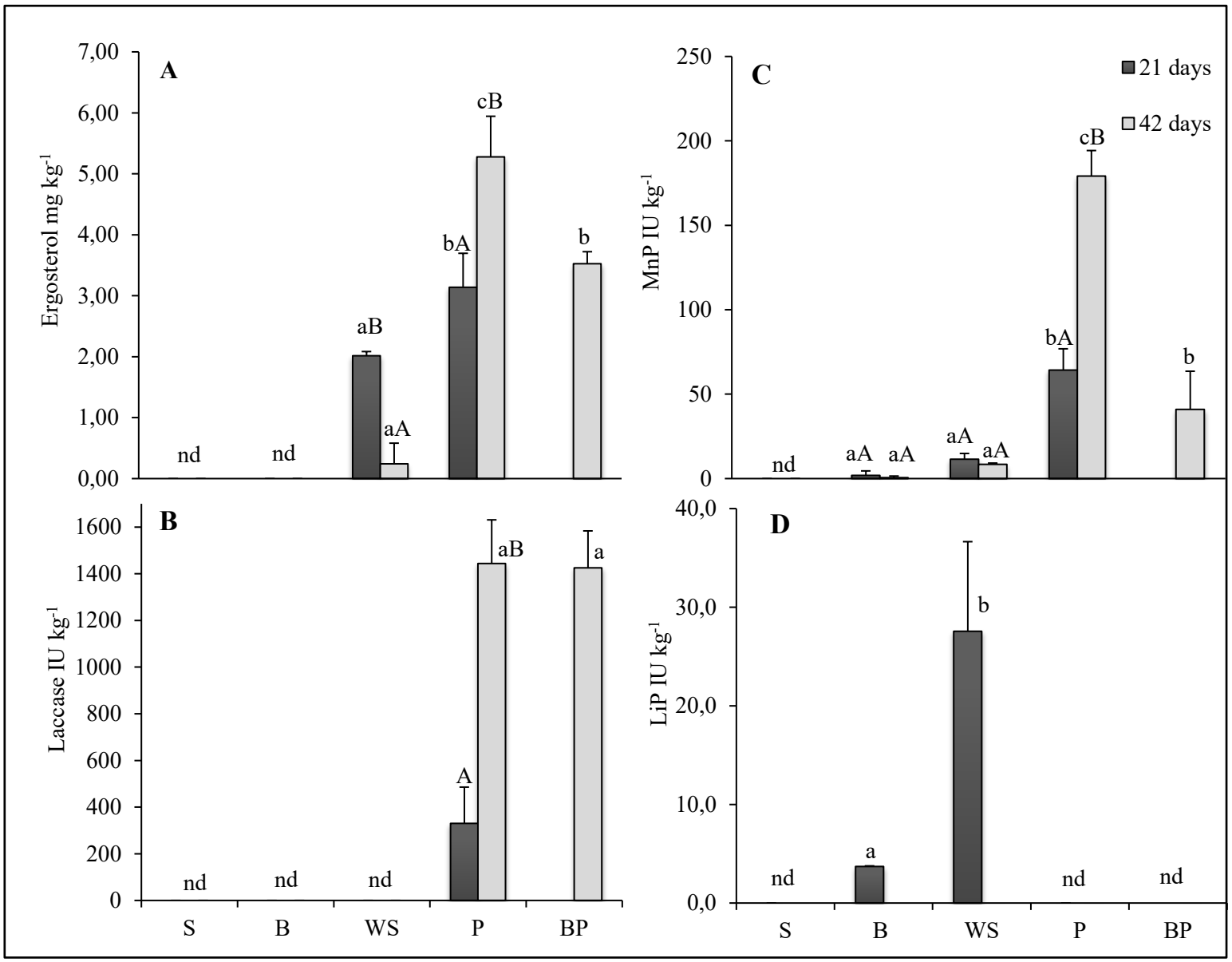
Different lowercase letters indicate significant differences between remediation treatments at same sampling time ($p < 0.05$). The asterisk (*) denotes significant differences between sampling times within the same treatment including B at 21d and BP at 42d ($p < 0.05$). † indicates significant differences between PAH degradation rate of P at 21d and BP at 42d ($p < 0.05$).

Table 2: Bioavailable PAH fraction (%) of initial subsoil, 21 and 42 days of incubation for each remediation treatment: non amended soil (S), biochart treatment (B), bioaugmentation treatment with wheat straw (WS), mycoremediation treatment with *P. ostreatus* (P) and sequential biochar amendment plus mycoremediation (BP).

PAH	Bioavailable PAH fraction (%)									
	Initial	S		B		WS		P		BP
	Soil	21d	42d	21d	42d	21d	42d	21d	42d	42d
Flu	93^{AB}	90	90^{AB}	90	96^{B}	88	87^{AB}	77	62^{A}	68^{AB*}
Phe	91^{AB}	91	92^{AB}	90	105^{B}	88	87^{AB}	87	72^{A}	91^{Ab}
Ant	83	86	84	82	89	75	78	62	69	54^{*}
Fla	77^{B}	78	78^{B}	77	81^{B}	77	69^{B}	71	37^{A*}	$42^{A*\dagger}$
Py	76^{CD}	78	78^{CD}	76	83^{D}	76	68^{C*}	70	31^{A*}	$45^{B*\dagger}$
BaA	66^{B}	71	72^{B}	69	72^{B}	76	71^{B*}	69	39^{A*}	58^{B}
Chr	56^{BC}	61	64^{C}	58	58^{C}	65	58^{C}	57	24^{A*}	43^{B}
BbF	28^{B}	35	36^{B}	29	26^{B}	36	26^{B*}	31	0^{A*}	9^{A}
BkF	37^{CD}	39	40^{D}	33	39^{CD}	37	30^{BC*}	33	8^{A*}	$21^{B*\dagger}$
BaP	29^{bBC}	34^{b}	33^{C}	28^{b}	27^{BC}	26^{b}	15^{B*}	11^{a}	0A	16^{B}
DBahA	11	8	4	0	0	0	0	0	0	10
BghiP	1	1	1	0	0	0	0	0	0	0
IcdP	11^{dC}	12^{d}	8^{BC}	1^{ab}	1^{AB}	$6b^{c}$	0^{A}	0^{a}	0A	1^{AB}
Σ3-rings	91^{AB}	91	91^{AB}	89	104^{B}	87	86^{AB}	86	71^{A}	89^{AB}
Σ4-rings	74^{B}	76	76^{B}	74	79^{B}	75	68^{B*}	70	34^{A*}	$45^{A*\dagger}$
Σ5,6-rings	25^{CD}	30	29^{D}	24	24^{CD}	28	20^{C*}	24	2^{A*}	$11^{B*\dagger}$
Σ13 PAH	78^{C}	79	78^{C}	77	84^{C}	77	71^{C}	72	42^{A}	$55^{B\dagger}$

Different lowercase letters indicate that differences between remediation treatments at 21 days of incubation were significant ($p < 0.05$). Different uppercase letters indicate that differences between remediation treatments at 42 days of incubation were significant ($p < 0.05$). The asterisk (*) denotes significant differences between sampling times within the same treatment including B at 21d and BP at 42d ($p < 0.05$). † indicates significant differences between PAH degradation rate of P at 21d and BP at 42d ($p < 0.05$).

Figure 1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5,00
4,00
3,00
2,00
1,00
0,00
nd
nd
aB
aA
bA
cB
b
B
Laccase IU kg-1
1600
1400
1200
1000
800
600
400
200
0
nd
nd
nd
A
aB
a
C
MnP IU kg-1
250
200
150
100
50
0
21 days
42 days
nd
aA
aA
aA
aA
bA
cB
b
D
LiP IU kg-1
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10,0
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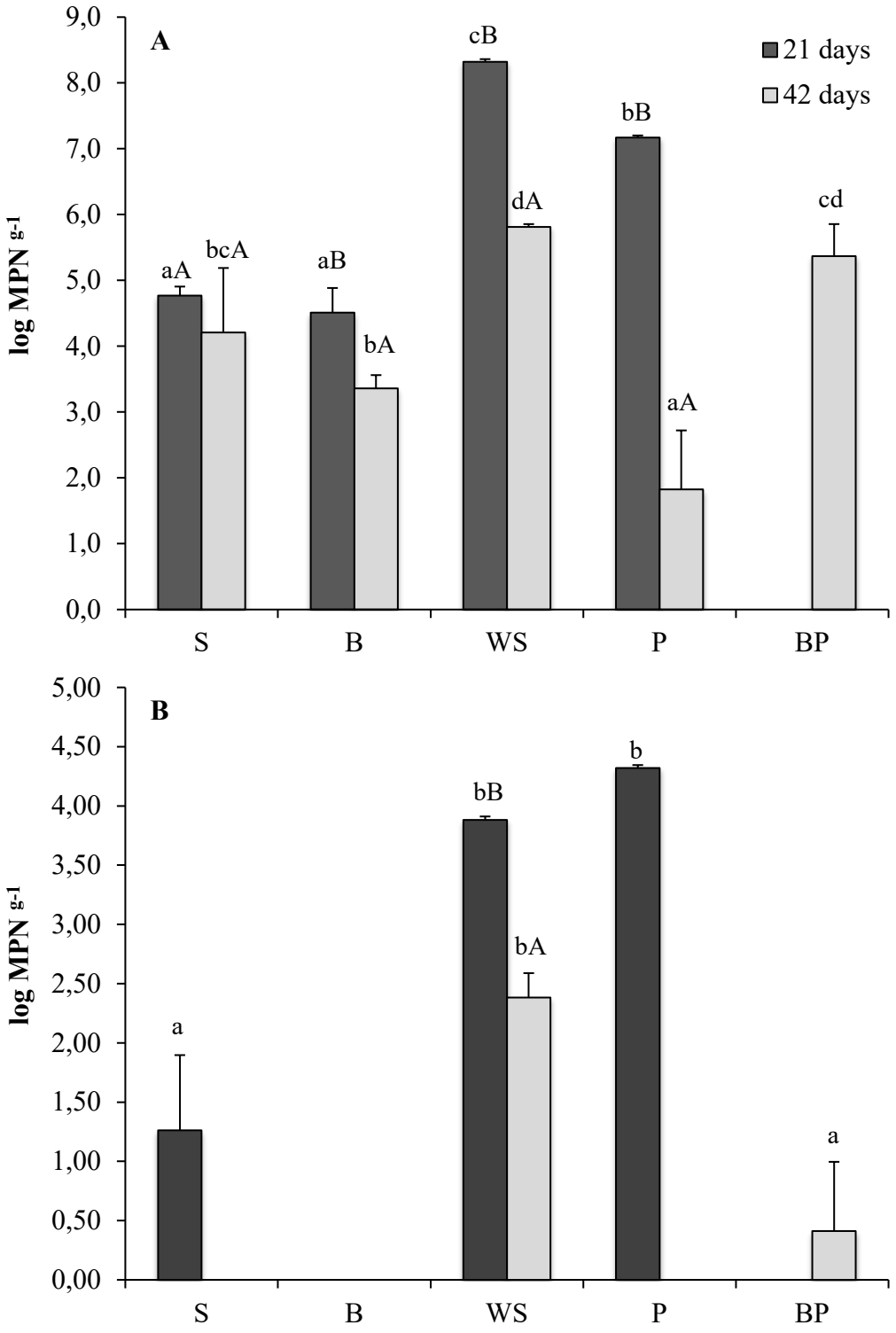
nd
a
b
nd
nd
S
B
WS
P
BP

Figure 2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21 days
42 days
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8,0
7,0
6,0
5,0
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3,0
2,0
1,0
0,0
aA
bcA
aB
bA
cB
dA
bB
aA
cd
S
B
WS
P
BP
B
log MPN g-1
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3,00
2,50
2,00
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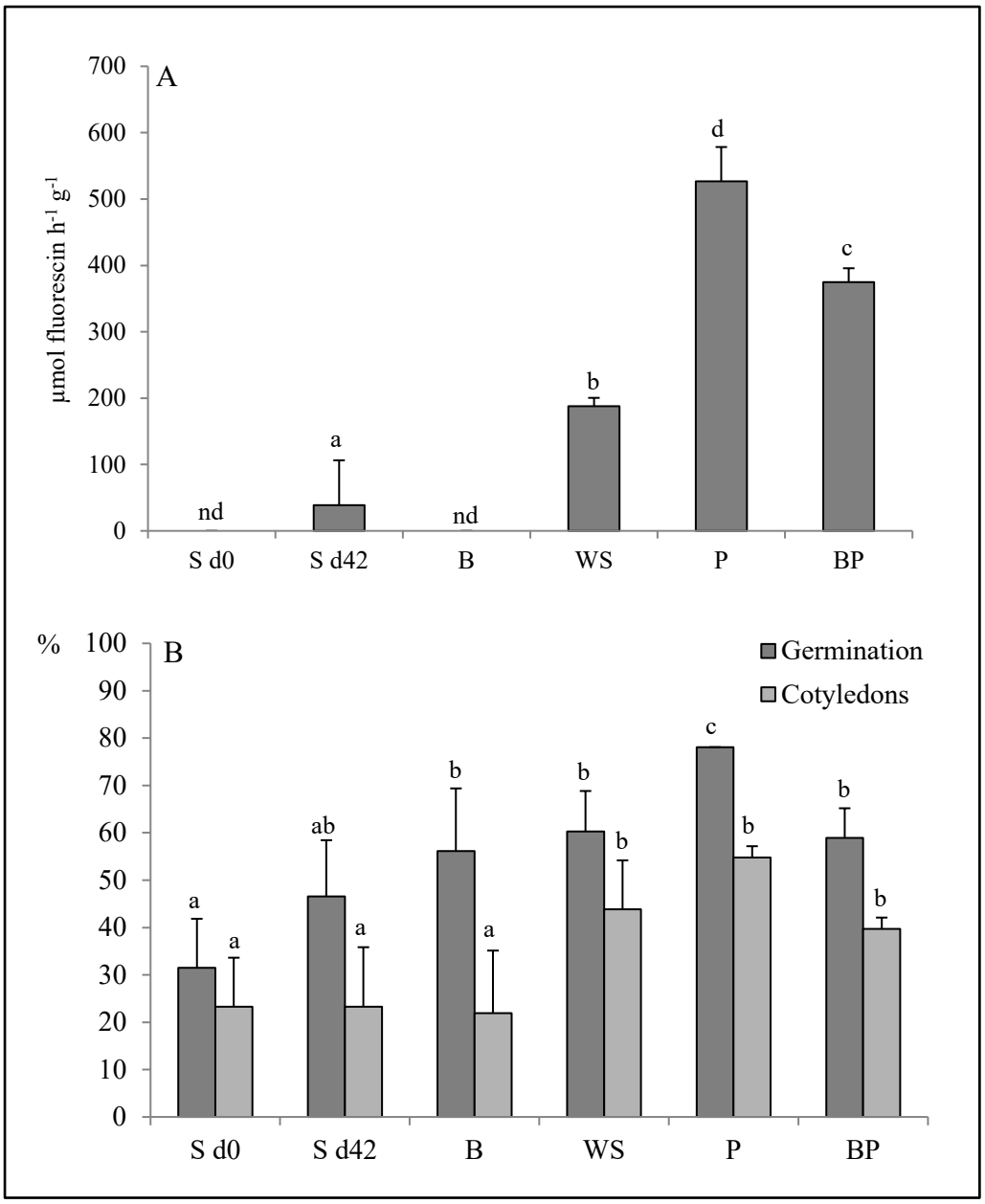
0,00
a
bB
bA
b
a
S
B
WS
P
BP

Figure 3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nd
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d
c
S d0
S d42
B
WS
P
BP
B
%
100
90
80
70
60
50
40
30
20
10
0
Germination
Cotyledons
a
a
ab
a
b
a
b
b
c
b
b
b
S d0
S d42
B
WS
P
BP