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Biochar Addition to a Mediterranean Agroecosystem: Short-Term Divergent Priming Effects

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Abstract: The goal of this study was to evaluate biochar's resistance to microbial decomposition and its impact on native soil organic matter (SOM) decomposition. Conducted in a vineyard with a sandy loam Mediterranean soil with neutral pH and low organic carbon content, the experiment involved the application of 6.5 g biochar kg^{-1} derived from pine (PB) and corn cob (ZB). The monitoring period spanned two years, with soil samples collected at short- and medium-term timepoints (2 and 26 months post-application) and incubated in the lab for an additional 250 days. Soil respiration, the CO_2-C isotopic signature, and dissolved organic carbon (DOC_{hw}) were assessed to identify potential priming effects (PE) and evaluate their persistence over two years. In the short term, biochar-induced priming effects were feedstock and pyrolysis temperature dependent, exhibiting negative priming in high-temperature wood biochar and positive priming in low-temperature grass biochar. The mechanism behind short-term positive priming was attributed to the higher labile organic carbon (OC) content in ZB compared to PB. In the medium term, initial strong priming effects shifted to slightly negative priming effects in both biochars, indicating the depletion of labile carbon fractions and the emergence of physical protection processes that mitigated priming.

Keywords: biochar; field study; SOC mineralization; soil incubation; soil respiration; stable carbon isotopes

1. Introduction

The biological activity of soils is often estimated by the release of CO_2 mostly resulting from organic matter decomposition either under field conditions [1] or laboratory incubations [2]. Organic amendments generally stimulate soil biological activity depending on their origin and stability degree (i.e., their resistance to decomposition). Fresh organic wastes are rich in easily biodegradable substances that induce high CO_2 emissions when applied to soil and can strongly modify soil microbiota communities [3]. On the contrary, stable organic amendments, such as mature compost, contribute to increases in soil organic matter content and enhance microbial activity. When the priority is to increase soil organic carbon stocks, stable organic materials, such as biochar, are preferred [4]. Biochar has garnered significant attention in recent years for its potential as a soil amendment [5]. Additionally, the incorporation of biochar into soil offers various environmental advantages, including long-term carbon sequestration [6], offsetting CO_2 emissions [7], and contributing to a broader global carbon-negative strategy [8,9].

Biochar results from the thermochemical carbonization of biomass in low oxygen concentration conditions and is used as a soil amendment [10]. Its highly aromatic and condensed structure confers high resistance to abiotic and biotic degradation [11]. These

properties explain the expected carbon sequestration by biochar application to soil [4]. However, the utility of biochar as a carbon sink has been largely discussed since some studies have shown that is able to promote native soil organic matter (SOM) decomposition [12,13] through the so-called positive priming effect (PE). This trend has been also observed in other organic or mineral amendments (such as fresh biomass or other organic or inorganic materials) [3–14], but alternative trends, such as neutral and negative priming effects (i.e., reduced native SOM decomposition), have been also reported elsewhere [3,15–17].

The manifestation of the priming effect (PE) in a biochar-amended soil is contingent on several factors, encompassing the characteristics of the biochar itself, such as its raw material, pyrolysis temperature, and residence time, along with the distinct microbial community and physicochemical properties inherent to the receiving soils. This complexity underscores the nuanced interplay of elements within biochar soil systems. Moreover, a negative correlation between biochar temperature production and PE has also been described [13,18–21]. Elevated production temperatures have been associated with higher levels of polycyclic aromatic compounds [22] and an enhanced sorption capacity [23], the latter contributing to reduced biochar decomposability. Conversely, low-temperature biochars have more easily degradable carbon structures that stimulate microorganism's activity and induce positive PE, as already shown in some studies [24,25]. Contrary to this finding, other authors have not identified a correlation between biochar pyrolysis temperature and the PE [26]. This implies that PE is likely influenced by various factors beyond biochar temperature production, such as the nature of the biochar feedstock [27–29], ecotoxicological risks [30], pH [13,18,31], aggregate composition [32,33], clay content and clay type [18,19], carbon content [32,34], the presence of plants [35], and environmental factors, such as temperature [36].

However, the priming effect is not a permanent state, as it depends on some properties that fluctuate over time, such as the accessibility of microorganisms to OM [37]. In addition, the application of fresh OM promotes fast decomposition and a "r strategy" in the microorganism communities present [3]. Subsequently, the residual and highly resistant organic matter (OM) undergoes a gradual decomposition, orchestrated by microbial communities employing a "K strategy" that steadily enhances their dominance over time in the decomposition process. This partly explains why, in general, a short-term positive PE and a later negative PE are found after the addition of biochar [34]; therefore, the duration of the experiment is important for the interpretation of results [19]. The surge in soil carbon decomposition observed shortly after the introduction of biochar arises from a small fraction of labile biochar carbon that can be degraded. Importantly, this phenomenon is not indicative of a genuine positive PE [21]. However, this observation does not contradict the long-term persistence of the majority of carbon within the biochar. As an example, [19] reported that labile and recalcitrant biochar carbon have a mean residence time of about 108 days (ca. 3%) and 556 years (ca. 97%), respectively. Similarly, in a two-year field study using stable isotopes in a savannah Oxisol [1], only 2.2% of the black carbon applied was lost through respiration over the experimental period.

The distinction between decomposition coming from biochar or native SOM pools can be achieved using two-component studies employing isotope techniques [38]. These techniques allow determining the respired CO_2 contribution from biochar and native SOM using isotope partitioning estimations. In addition, three-component studies have been used for this purpose, adding a third isotopic signature to the assessment, such as in biochar-amended soils with plants biomass [35,39–41] or with organic wastes [12]. Most of these studies have shown that biochar suppresses decomposition of added biodegradable materials that are different to native SOM. An explanation is that biochar might provide physical protection of this newly added OM by increasing soil aggregation [41].

However, very little research exists on biochar-amended Mediterranean agroecosystems under field conditions in soil with chronically low organic matter content and with discontinuous decomposition throughout the year. Furthermore, most studies on this topic use short-term incubation that prevents long-term projections [34]. Our case study focuses

on a vineyard, which is one of the most prevalent crops in Spain and other Mediterranean countries. According to the Ministry of Agriculture, Fisheries, and Food, as of 31 July 2020, the potential vineyard production in Spain reached 989,279 hectares. The widespread cultivation of this crop positions it as a promising candidate for potential use in carbon farming, a crucial tool in the battle against climate change.

The aim of this work was to assess the resistance of two contrasted biochars to microbial decomposition and to determine how its application influences short- and medium-term native SOM decomposition under a Mediterranean climate. The study was conducted over a two-year period in a vineyard with soil amendments using two distinct biochars. The experiment was based on laboratory incubations of field-collected samples using CO_2-C stable isotopes as tracers in order to (1) identify significant and contrasting PE effects for the studied biochars in the short term and (2) determine the eventual persistence of those effects in the medium term (after two years).

2. Materials and Methods

2.1. Site and Soil Description

The study was conducted in a Fluventic Haploxerept soil [10,42] in a 20-year-old Mediterranean vineyard located at Vimbodíi Poblet (Catalonia, NE, Spain). Its organic carbon and total nitrogen contents were low (9.7 and 0.89 g kg^{-1} soil, respectively [43]). Microbial biomass C, estimated as the ATP/biomass-C ratio, calculated as described in [44] was also low (49.9 mg C kg^{-1} soil). The vineyard adhered to ecological agriculture practices and had received composted cow manure as fertilizer two years prior to the commencement of this study. However, no additional fertilization was applied during the experimental period. The only fresh organic matter input was derived from vineyard pruning remains and leaves. Additionality, no agrochemicals were used with the exception of the Bordeaux mixture treatments applied for fungal disease control. The vineyard underwent ploughing three to four times per year to control weeds.

2.2. Biochar Characterization

Two different biochars were tested in the study. The first biochar, labelled as PB, was produced through gasification at 600–900 °C with a moderate residence time (10–20 s [45]). It was produced from a blend of pine wood splinters (*Pinus radiata* and *Pinus pinaster*). The second biochar, named ZB, resulted from the slow pyrolysis of corn cobs (*Zea mays*) at 450–500 °C, with an extended residence time (5–30 min [45]). Prior to field application, both biochars underwent a sieving process to achieve a particle size of 2 mm, and a subsample of the field batch was grounded for analysis. The δ^{13}C values for ZB and PB were 28.03‰ and 13.12‰, respectively [43].

2.3. Experimental Design and Sampling

Plots, each consisting of a 10 × 8.8 m^2 area and containing 40 *Vitis vinifera* plants, were randomly distributed throughout the vineyard. Three different treatments (in triplicate) were applied: control soil (S), soil amended with pine biochar (S + PB), and amended with corn cob biochar (S + ZB). Biochar was applied at a rate of 5 Mg C ha^{-1} of biochar (corresponding to 6.5 g kg^{-1} in the <2 mm soil fraction) at the range of many experimental biochar trials, as reported by [46], and applied once. The biochars were uniformly distributed on the soil surface and then incorporated into the arable layer by two successive plows at a 15 cm depth. Soil samples from each plot were collected in July 2013 and July 2015, after 2 and 26 months, respectively, of the biochar application. Each sample corresponded to a composite sample, comprising eight 4 dm^3 soil cores randomly taken (approximately 45 kg per sample) and corresponding to the top 10 cm of the arable layer. Soil samples were sieved to 5 mm in the field to separate gravels, then sieved again to 2 mm in the lab, and stored at 4 °C. A representative portion of each sample was grounded and sieved to 0.02 mm to carry out some of the analyses. Laboratory analyses were carried out in triplicate for each sample.

2.4. Carbon Dioxide Released from Soil Samples

The evolution of organic carbon as CO_2 in the soil samples was monitored over a 250-day period in the laboratory. To achieve this, field-fresh soil samples were meticulously re-moistened to 50% of their maximum water-holding capacity according to [2]. Subsequently, 40 g of the re-moistened soil sample was placed within a 150 mL polyethylene container positioned inside a 1-L jar alongside a CO_2 trap. The trap, consisting of 5 mL of 0.2 M sodium hydroxide (NaOH) in a 50 mL polyethylene cup, was accompanied by an additional cup containing deionized water to maintain saturated humidity. The jars were hermetically sealed and stored in a dark room at 25 °C for a duration of 250 days. A control jar without soil samples was employed to account for the background concentration of CO_2-C. The NaOH solution was periodically replaced following a geometric schedule (more frequently in the initial months and more spread over time in the later months, see Figure S1). The CO_2-C evolved from microbial respiration was measured directly in the 50 mL polyethylene container through back-titration with 0.05 M HCl, using phenolphthalein as indicator, after adding 15 mL of 0.05 M $BaCl_2$ solution.

To evaluate the isotopic $\delta^{13}C$ signature of the evolved CO_2–C from the same soil samples, additional replicates were prepared as outlined earlier. These replicates were then kept in the dark at 25 °C for a duration of 250 days within a sealed 1 L jar equipped with a lid featuring a rubber septum. This setup allowed for two sampling instances (after 30 days and at the end of the 250-day incubation period) by extracting 10 mL of cumulative air using a syringe. The collected air samples were subsequently transferred to vacutainers and injected into an isotope ratio mass spectrometer (IRMS) (MAT253, Thermo Electron Corporation, Bremen, Germany) in continuous flow mode, employing the sample introduction system GasBench II (Thermo Electron Corporation, Bremen, Germany).

2.5. Hot Water Extractable Carbon

Dissolved organic carbon (DOC_{hw}) was extracted following [47] with some modifications. Briefly, eight grams of fresh soil (stored at 4 °C) was placed in 50 mL PTFE centrifuge tubes and subjected to extraction with 20 mL of distilled water (1:2.5 *w/v*). The extraction process involved one hour of shaking at room temperature on a vertical shaker set at 30 rpm. Subsequently, the samples were left to stand for 1 h at 80 °C in a hot-water bath, shaken for 10 min, and then centrifuged at 10,000 rpm for 10 min. Following centrifugation, the supernatants were decanted and filtered through Whatman® #40 filter paper (Whatman Inc., Brentford, UK). This procedure was carried out in duplicate, once to quantify dissolved organic carbon and the other to determine the isotope signature.

The determination of dissolved organic carbon (DOC_{hw}) involved potassium dichromate oxidation, employing the following steps: a 4 mL aliquot of the supernatant was placed in a Pyrex tube and oxidized in a mixture of 0.5 mL of 66.7 mM $K_2Cr_2O_7$ and 4 mL of a biacid mixture (H_2SO_4/H_3PO_4 in a 1:1 *v/v* ratio). The tubes were vortexed for 15 s and heated to 150 °C in a digestion block for 10 min. After cooling, the solution was transferred to an Erlenmeyer flask and rinsed with 90 mL of distilled water. The excess dichromate ($Cr_2O_7{-2}$) was back titrated with 33.3 mM of ammonium iron (II) sulfate (Mohr's salt) using diphenylamine as an indicator.

For the analysis of the carbon isotope signature of the dissolved organic carbon in hot water (DOC_{hw}), the hot water extracts underwent filtration through a hydrophilic glass fiber filter with a pore size of 0.7 μm. Subsequently, they were transferred to ceramic evaporation capsules for concentration at 80 °C until approximately one milliliter remained. Throughout this process, the ceramic capsules were gently agitated to facilitate sample concentration. The concentrated extract was then transferred to a tin capsule designed for liquids and heated at 80 °C until the sample was completely dry. The isotopic signature of the DOC_{hw} was determined using a Flash EA 1112 (Thermo Electron) analyzer at 1020 °C.

2.6. Quantification of the Carbon Contribution Attributable to Biochar (f_B) and Soil (f_S) Using Isotope Partitioning

The stable isotopic partitioning was assessed through a two-compartment model, according to the approach of [35,38], in order to quantify the relative percentage of evolved CO_2-C attributable to soil and biochar. This approach assumes that the $\delta^{13}C$ of the biochar-amended soil ranges between that of the biochar and that of the native organic matter depending on the partial contribution of each component, which is estimated as follows:

Equation (1) Biochar-C fraction (%)

$$f_B(\%) = \left(\frac{\delta^{13}C_{S+B} - \delta^{13}C_S}{\delta^{13}C_B - \delta^{13}C_S} \right) \times 100 \tag{1}$$

f_B: evolved carbon fraction attributable to pine or corn cob biochar in the biochar-amended soil (S + PB or S + ZB)
$\delta^{13}C_{S+B}$: $\delta^{13}C$ of evolved CO_2 from pine or corn cob biochar-amended soil (S + PB or S + ZB) in each sampling time for each incubation period
$\delta^{13}C_S$: $\delta^{13}C$ of evolved CO_2 from control soil (S) in each sampling time for the two incubation periods
$\delta^{13}C_B$: $\delta^{13}C$ of pine or corn cob biochar (PB or ZB)

The percentage of the native soil-C fraction evolved during incubation was quantified by subtracting the biochar-C fractioning of each sampling time at each incubation period in biochar amended soils (Equation (2)). The amount of evolved native soil CO_2-C was calculated by multiplying the native soil carbon fraction ($f_S(\%)$) (Equation (2)) by the total carbon evolved in each sampling time during two incubation periods in biochar-amended soils.

Equation (2) Native soil-C fraction (%)

$$f_S(\%) = 100 - f_B(\%) \tag{2}$$

f_B: evolved CO_2-C fraction attributable to pine or corn cob biochar in the biochar-amended soil (S + PB or S + ZB) for each sampling time of each incubation period
f_S: evolved CO_2-C fraction attributable to native soil in the biochar-amended soil (S + PB or S + ZB) for each sampling time of each incubation period (Equation (1)).

The amount of evolved CO_2-C from control soil (S) was subtracted from the amount of evolved CO_2-C of native soil-C in biochar-amended soils (S + PB or S + ZB) to quantify priming-C effects for each sampling time of each incubation period.

2.7. Data Analysis

Before statistical analysis, the data underwent normality testing using the Shapiro–Wilk test. For evolved CO_2-C and DOC_{hw}, global significance tests of the effects of biochar amendment (S, S + PB, S + ZB) were performed using two-way repeated measure ANOVA. Significant differences between biochar amendment within a sampling time and between samplings within a biochar amendment were assessed using Bonferroni tests at a probability level of 0.05. For isotopic signature analysis, the values of soil amended with biochar (S + PB and S + ZB) were compared separately with control soil (S). Significance tests to assess the effects of biochar type were performed by comparing the isotopic signature of S + PB and S + ZB with S using two-way repeated measure ANOVA. After, S + PB and S + ZB were compared with S within a sampling time using Bonferroni tests at a probability level of 0.05. All statistical tests were performed using R Statistical Software v4.2.0 (2022-04-22 ucrt) (R Foundation, Indianapolis, IN, USA).

3. Results

3.1. CO_2-C Evolved from Soil Incubation

During the 250-day incubation of the July 2013 samples, the initial CO_2-C production was high during the first 30 days of incubation and followed by a slowdown, while this trend was not observed in the 2015 sample (see Figure S1 in the Supplementary Materials). Figure 1 shows the mean CO_2-C release day^{-1} in the 0–30 day and 90–250 day incubation period, showing that respiration rates were higher in the 2-month sample (July 2013) than in the 26-month sample (July 2015).
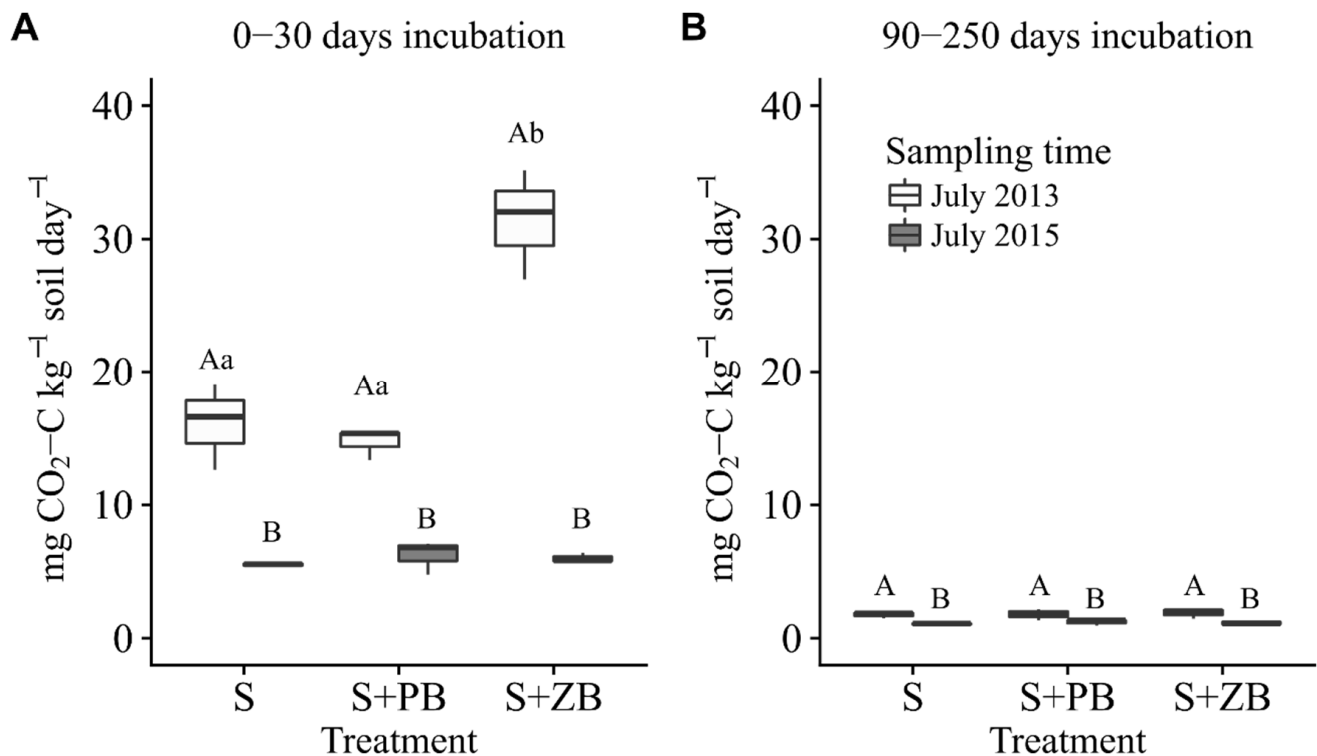
Figure 1. Mean CO_2-C day $^{-1}$ produced during the incubation of the field samples collected in July 2013 and 2015 (2 and 26 months after the biochar application, respectively): (**A**) between 0 and 30 days of incubation and (**B**) between 90 and 250 days of incubation in the control soil (S) and in soil treated with pine (S + PB) or corn cobs (S + ZB) biochar. Values with the same capital letters indicate the lack of significant differences between sampling times within treatments ($p < 0.05$), while equal small letters imply the lack of differences between treatments within sampling times ($p < 0.05$). No significant differences were found when letters are not present.

In the July 2013 samples, the CO_2-C evolved at 30 days from S + ZB samples was the only value significantly higher than control (S) and also higher than the S + PB treatment ($p < 0.001$). However, no differences were observed in the 90–250 day period. In the July 2015 samples, no significant differences between treatments were observed. Globally, total CO_2-C release during the incubations decreased by nearly a half in 2015, two years after the biochar application (Table 1).

Regarding the percentage of CO_2-C to TOC, in general, a significantly higher percentage was observed in S compared to the biochar-added plot, while similar percentages were found in both biochar-amended soils ($p < 0.01$) (Table 1). When the percentage of TOC released as CO_2-C over 250 incubation days was calculated, we observed that the percentage was nearly twice as high in biochar-treated plots than in controls in both 2013 and 2015 samples ($p < 0.01$) (Table 1).

Table 1. Soil total organic carbon (TOC) [43] and percentage of CO_2-C to TOC in a soil sample treated with pine biochar (S + PB) and corn cob biochar (S + ZB) compared to control (S) at two sampling times (July 2013 and July 2015) after biochar application. Mean values and standard deviation of three replicates are shown. Values sharing small letters indicate the lack of significant differences between treatments within sampling times ($p < 0.05$).

| Biochar Treatment	Total Organic Carbon (TOC) (g kg^{-1})	CO_2-C/TOC (%)
July 2013		
S	9.77 ± 0.54 a	6.6 a
S + PB	16.87 ± 2.56 b	3.9 b
S + ZB	20.21 ± 2.37 b	5.3 a
July 2015		
S	9.80 ± 0.85 a	3.7 a
S + PB	15.97 ± 1.23 b	2.5 b
S + ZB	15.64 ± 2.03 b	2.5 b

3.2. Dissolved Organic Carbon

The two biochars in this study had contrasting hydrophobicity (direct observation), with PB being highly hydrophobic compared to ZB, which was more hydrophilic. In agreement, the dissolved organic carbon (DOC_{hw}) concentration of PB was 0.29 ± 0.07 g kg^{-1} of biochar and that of ZB was 9.50 ± 3.30 g kg^{-1}. When the DOC_{hw} measurements were performed in soils, the values ranged between 100 and 170 mg kg^{-1} (Figure 2), with significantly higher values in ZB biochar plots compared to PB biochar plots ($p = 0.01$). Moreover, no significant differences in DOC_{hw} were observed between sampling times (July 2013 and July 2015) within each treatment (Figure 2).
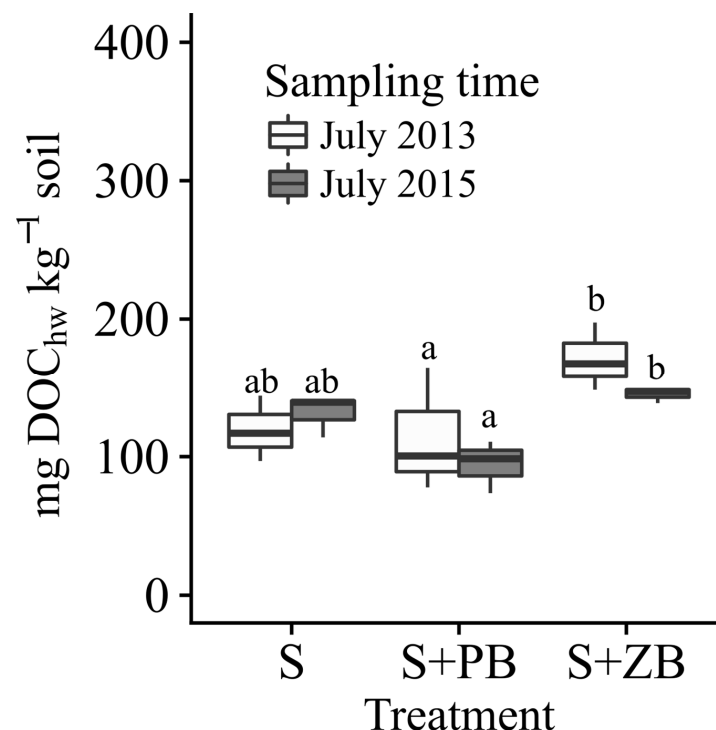
Figure 2. Hot water dissolved organic carbon (DOC_{hw}) content of a control soil (S) sample and soil samples treated with pine (S + PB) or corn cob (S + ZB) biochar at 2 months (July 2013) and 26 months (July 2015) after biochar application. Values with the same small letters imply the lack of differences between treatments within sampling times ($p < 0.05$). No significant differences were observed between sampling times within each treatment.

When the ratio between the CO_2-C released over 30 days to DOC_{hw} was calculated, we observed that two months after biochar addition (July 2013 samples), the carbon released as CO_2 was nearly three times the DOC_{hw}, whereas similar amounts were found two years after (July 2015) (Figure 3). The CO_2-C/DOC_{hw} ratio values between sampling times were clearly significant ($p < 0.001$), but no differences between treatments were observed.
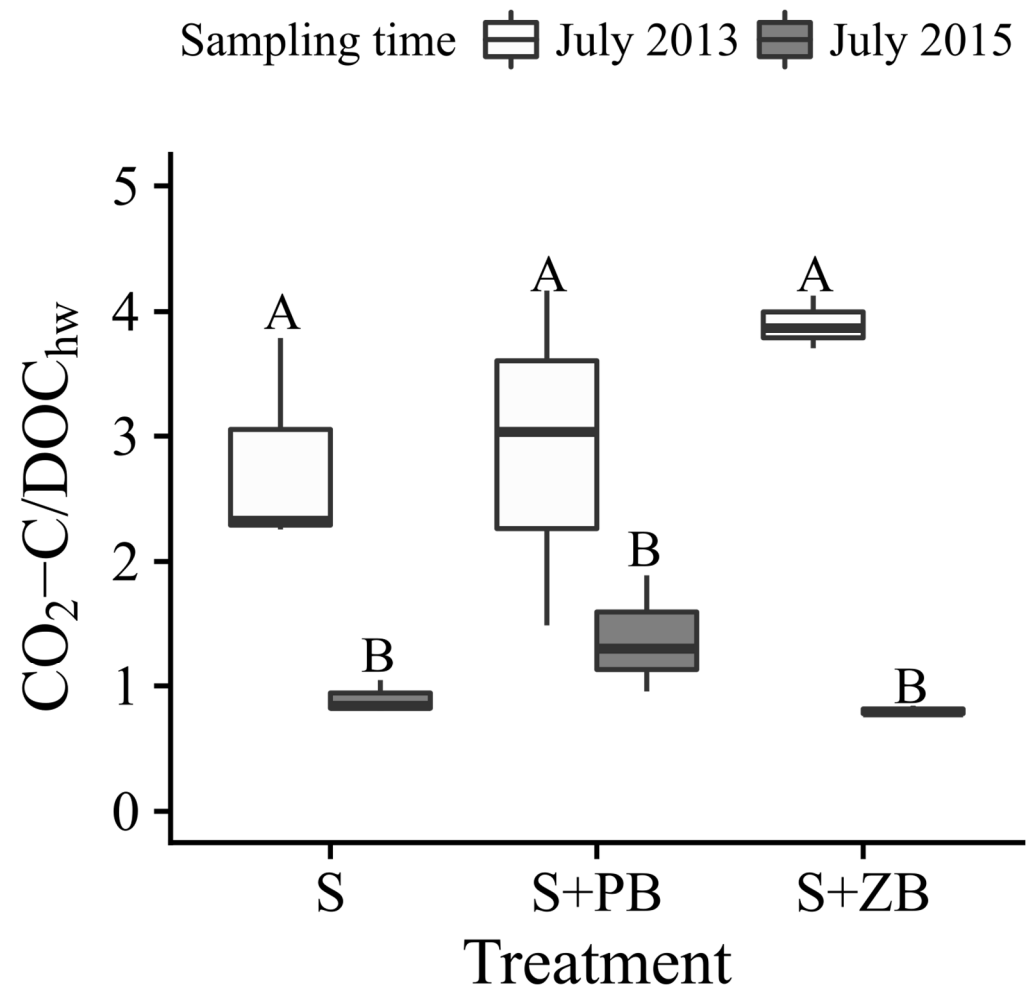
Figure 3. Ratio between cumulative CO_2-C respired over 30 days and DOC_{hw} of the control soil (S) sample and soil samples treated with pine (S + PB) or corn cob (S + ZB) biochar at 2 months (July 2013) and 26 months (July 2015) after biochar application. Values with the same capital letters imply the lack of differences between a sampling time within treatments ($p < 0.05$). No significant differences were observed between treatments within sampling times.

3.3. Carbon Isotopic Signature

The isotopic signatures of hot water dissolved organic carbon (DOC_{hw}) and that of the CO_2-C released in the July 2013 and the July 2015 samples were assessed.

3.3.1. δ^{13}C of the Dissolved Organic Carbon

Similar δ^{13}C values were found for the DOC in all treatments (Figure 4). However, slightly higher values were observed in S + PB compared with S ($p = 0.02$), but no significant differences were detected between S and S + ZB despite this biochar having a higher δ^{13}C ($-13.12‰$ for ZB biochar). The δ^{13}C range agrees with the dominance of dissolved organic carbon derived from C_3 plants, even in the S + ZB treatment. Significant differences were observed between δ^{13}C of DOC_{hw} and that of whole soil C in all treatments.
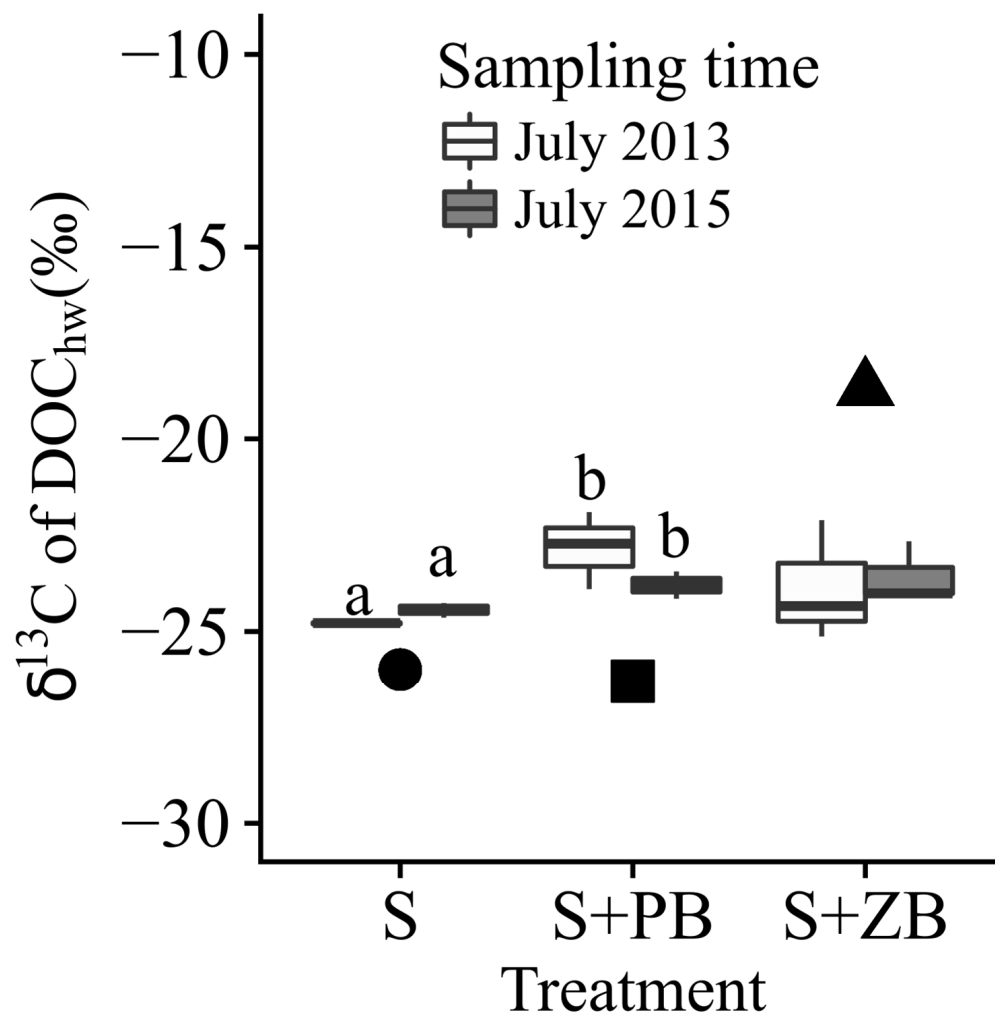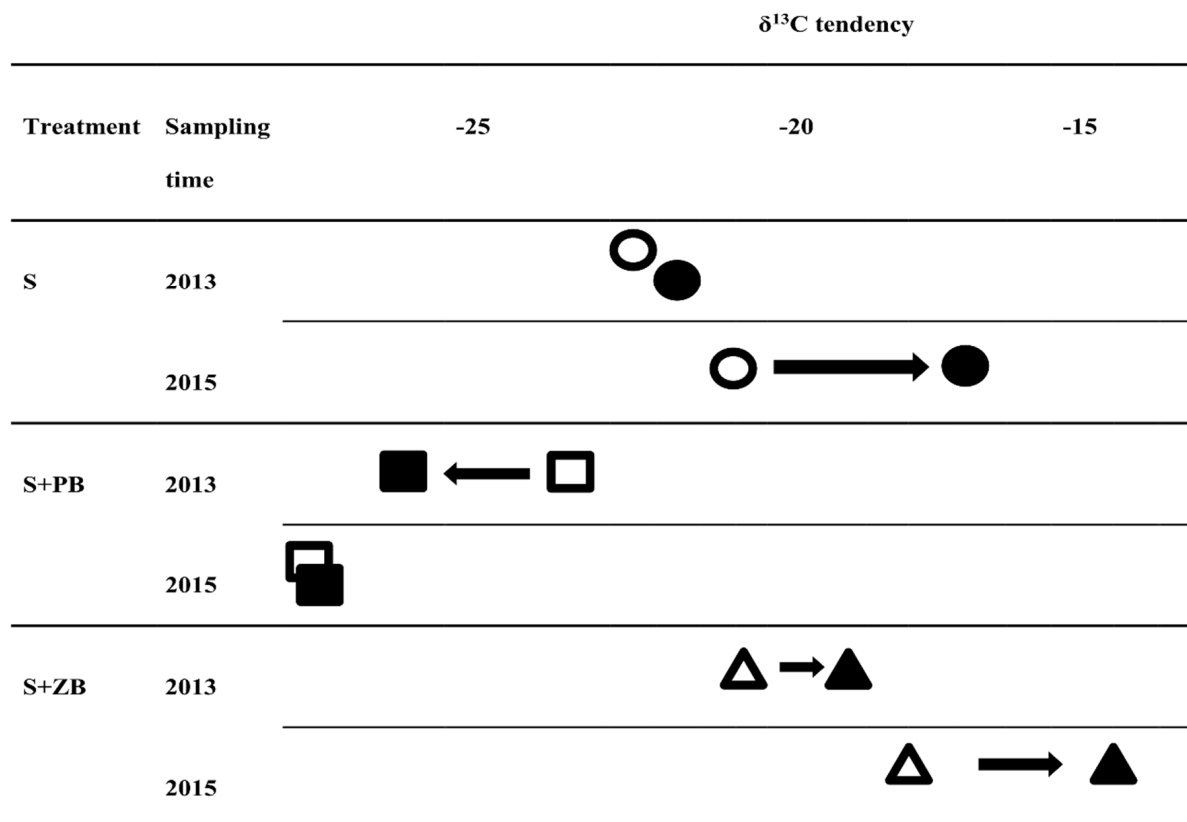
Figure 4. $\delta^{13}C$ of hot water dissolved organic carbon (DOC_{hw}) of a control soil (S) sample and soil samples treated with pine (S + PB) or corn cob (S + ZB) biochar at 2 months (July 2013) and 26 months (July 2015) after biochar application. Circles represent the mean $\delta^{13}C$ of whole control soil (S), squares represent S + PB soil $\delta^{13}C$, and triangles represent S + ZB soil $\delta^{13}C$. Values with the same small letters imply the lack of differences between treatments within sampling times ($p < 0.05$). No differences between sampling times were observed.

3.3.2. $\delta^{13}C$ of Released CO_2-C

Regarding the $\delta^{13}C$ of the CO_2 released from the samples, no significant differences were found between treatments (S and S + PB or S and S + ZB). Similarly, no significant differences were observed between the $\delta^{13}C$ of CO_2 respired during the first month or over 250 days of incubation from soil samples collected in July 2013, with values around −23‰. However, looking at the mean $\delta^{13}C$ values, there is trend for higher mean values in July 2015 in the S and S + ZB treatments (below −20‰) when compared to those of July 2013, while the opposite tendency was observed for S + PB (Figure 5), where $\delta^{13}C$ values in 2015 samples were lower compared to 2013. The $\delta13C$ values from soil samples taken in July 2015 further support the same trend, comparing the 30-day and 250-day incubation periods between S and S + ZB treatments. Notably, there were no discernible changes observed in S + PB soil samples in July 2015.

δ¹³C tendency

Treatment	Sampling time	-25	-20	-15
S	2013		○ ●	
	2015		○ ⟶ ●	
S+PB	2013	■ ⟵ □		
	2015	◪		
S+ZB	2013		△ ⟶ ▲	
	2015			△ ⟶ ▲

Figure 5. Changes of δ^{13}C tendency of evolved CO_2-C from the control soil (S) sample and soil samples treated with pine (S + PB) or corn cob (S + ZB) biochar at two sampling times (July 2013 and July 2015) measured after 30 days (white symbols) or 250 days (black symbols) of incubation. Circles, squares, and triangles correspond to the isotopic signature of control soil (S) and the S + PB and S + ZB treatments, respectively.

3.4. Priming Effects of Biochar on Native Soil Organic C

Using the δ^{13}C mean values in Figure 5 and Equation (1), we estimated that the relative contribution of each biochar to the CO_2-C released was relatively low and similar (17% and 18% in S + PB and S + ZB, respectively) after 30 days of incubation of the July 2013 soil samples. In addition, the CO_2-C fraction from both biochars tended to increase during the laboratory incubation from day 30 to day 250 in the July 2013 (from 17% to 59% in S + PB and 18% to 29% in S + ZB) and the July 2015 samples (from 77% to 79% in S + PB and 36% to 71% in S + ZB). When the relative contribution was expressed as true CO_2-C release, which is calculated as the product of f_B (Equation (1)) and evolved CO_2-C (Figure 1), we observed a decrease from the 30-day incubations (ca. 0.15 mg of biochar-C kg^{-1} day^{-1}) to the 250-day incubations (ca. 0.02 mg of biochar-C kg^{-1} day^{-1}) in both biochars.

During the 30-day incubation of the 2013 samples, native C mineralization in S+ZB (obtained by subtracting the CO_2-C released in controls to the CO_2-C derived from native organic matter in biochar treated samples, the last estimated by using the Equation (2)) was higher than control soil (positive priming) while the opposite tendency was found in S + PB (negative priming) (Figure 6). Conversely, in the 30-day incubations from the 2015 samples, a lower native C mineralization (negative priming) was observed in both biochar amended soils compared to control soil. However, negligible changes between control and biochar treatments were found in the 250-day incubations at any sampling time (Figure 6).
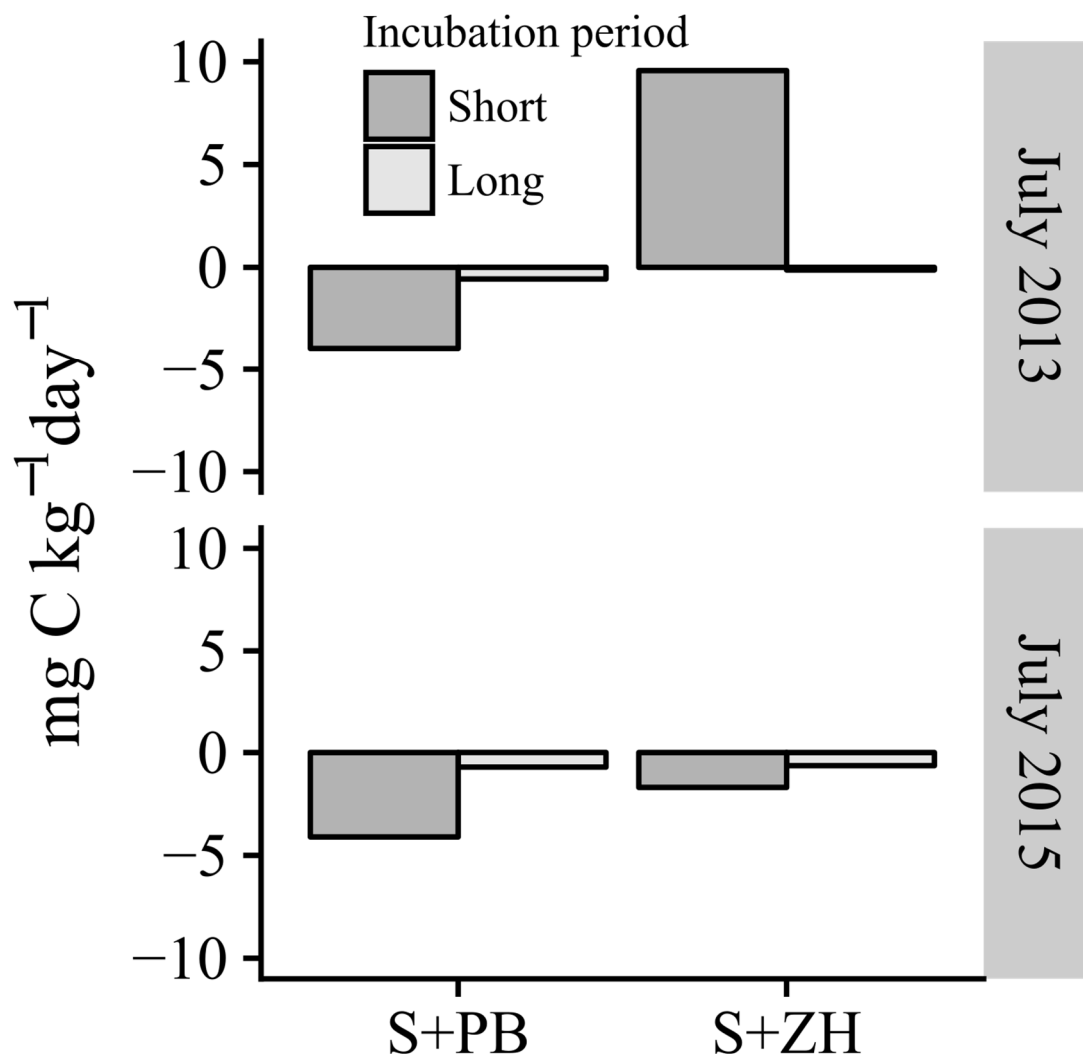
Figure 6. Mineralization balance of organic C in biochar treatments (S + PB, S + ZB) compared with control soil (mg CO_2-C kg^{-1} soil). Data were obtained from the cumulative CO_2-C at two incubation times (30-d and 250 d) of the field samples taken in July 2013 and July 2015 (2 and 26 months after biochar addition, respectively).

4. Discussion

The addition of fresh organic matter, such as plant debris, compost, or manure, to soils promotes the fast grow of specialized microorganisms and results in an initial increase in carbon mineralization [3] reflected as higher CO_2-C release. However, this is not always the case for biochar amendments, considered a very stable source of organic C. The evolved CO_2-C in soils and soils amended with biochar ranged between 1.89 and 28.57 mg kg^{-1} day^{-1} [13,48,49] or 0.5–8 mg kg^{-1} day^{-1} [13,26,50], respectively, with the results from our study close to this range (0.94–35.15 mg kg^{-1} day^{-1}). Despite this expected low contribution of biochar carbon to CO_2-C release, a small fraction of labile biochar components is prone to decompose and could increase global CO_2-C. This could occur without affecting native soil organic carbon, as reported by [21], or enhance or decrease its decomposition, in the so-called priming effect (PE). In our study, we found that although around 17% of the CO_2-C released came from the labile fraction of the biochars, two contrasting priming effects were observed for both of the biochars assessed.

4.1. Biochar Origin and Production Temperature Explain the Short-Term Priming Effects Observed

Priming effects in biochar-amended soils, defined as the change in native organic carbon decomposition as a result of biochar application, have been widely reported in the

current biochar literature [19,34], with both positive and negative effects, i.e., increases or decreases in the native SOC decomposition, reported [51]. Different factors could affect priming, with biochar type being one of the most influential [19,34,51,52]. On one hand, the addition of labile carbon from the biochar application could induce an increase in microbial activity, promoting SOC decomposition. However, on the other hand, the sorption of other organic compounds or changes in the soil's chemical (e.g., pH) and physical (e.g., increased soil aggregation) properties could suppress SOC mineralization [34–51]. In our study, we observed both negative and positive priming after 30 days of incubation of the 2013 samples with pine biochar (S + PB) and maize biochar (S + ZB), respectively (Figure 6). The positive priming observed in ZB was coupled to higher carbon mineralization in S + ZB plots (Figure 1) that was twice that of control or S + PB plots, primarily originating from the mineralization of native soil organic matter. CO_2-C released from biochar accounted for around 17% of the total released. In contrast, the opposite trend was observed in S + PB in the same sampling period, with a clear negative priming. According to [27,28,51], positive priming is expected in grass-derived biochars but also in biochars produced at low temperatures [34,52]. This finding is consistent with our study findings since ZB corresponds to a corn cob biochar produced by slow pyrolysis at 450–500 °C, and the pine wood biochar (PB) was produced at high temperatures of 600–900 °C. This feedstock and production temperature effect has been reported in other similar studies, showing suppression in native organic carbon losses of hardwood and pine biochars by [27,28], respectively. Contrasting with this generalized trend, positive priming of a *Pinus ponderosa* biochar has been reported [29], while other authors have described negative priming effects using grass materials [35,41]. Thus, in summary, our results highlight that the short-term priming effects depend principally on the feedstock and pyrolysis temperature used for its production, in agreement with other studies [19,34,51,52].

4.2. Short-Term Priming Effects Observed and Underlying Process

A variety of processes and mechanisms are proposed to explain the priming effects reported after biochar addition. In a meta-analysis, [34] identified the labile fraction of biochar as the main explanation for the positive PE due to its capacity to trigger the activity of soil microorganisms and subsequently speeding up the use of native organic matter. Moreover, in accordance with [51], positive PE occurs when the introduced substrate lacks specific nutrients, such as nitrogen, prompting an escalation in the decomposition of SOM to compensate for the deficiency. Additionally, when a complex substrate is introduced, SOM can undergo co-metabolism by microorganisms, which is facilitated by various hydrolytic enzymes. Thus, in our study, the positive priming potential of biochar was assessed by measuring some proxy properties that have been associated to a higher content in labile organic matter: (i) the labile carbon fraction in biochars, measured by calculating the carbon isotopic signature of the CO_2 released in soil–biochar mixtures; (ii) hot water-soluble carbon, a measure of the most labile fraction of biochar and soil–biochar mixtures, was assessed.

Regarding the labile fraction of biochar, mineralization tends to be enhanced in soils with low carbon content when utilizing biochar produced at low temperatures compared to biochar produced at high temperatures [51]. Moreover, in a previous study [43], we showed that the ZB contained a higher content in labile carbon compared to PB as measured using four chemical methods (strong and mild potassium dichromate oxidation, peroxide oxidation, and acid hydrolysis, see Table 2 in [43]). This agrees with the faster decomposition rates in ZB-amended soils during the 30-day incubation (0.9 g kg^{-1} of biochar day^{-1}, see Section 2.6) of the initial field samples compared to PB-amended soils (0.06 g kg^{-1} of biochar day^{-1}, see Section 2.6). These values are similar to those reported by [35] for a 47-week greenhouse experiment, where 0.6 g kg^{-1} of biochar day^{-1} was lost during the first week. These biochar decomposition rates are higher than those reported in other studies, such as [40], reporting a mean biochar decomposition rate of 0.17 g kg^{-1} in *Oryza sativa* biochar, and [39], reporting similar values (0.12 g kg^{-1}).

Another measure of carbon lability is hot water dissolved carbon (DOC_{hw}) of biochars, which is highly correlated with biomass and activity of microbes [47,53]. The DOC_{hw} was higher in ZB (9.5 ± 3.30 g C kg^{-1} biochar) compared to PB (0.29 ± 0.07 g C kg^{-1} biochar), agreeing with the chemical stability measurements reported in the previous paragraph. In agreement, in biochar plots, the significant increase in DOC after biochar application reported in other studies [54,55] was only shown in ZB-amended plots (Figure 2). This mostly arises from the higher DOC_{hw} content in this biochar but is also attributed to its positive priming effect that promotes the decomposition of native organic matter by microbes. Alternatively, the negative PE could be associated with the enhanced physical protection of native organic matter facilitated by biochar through the higher hydrophobicity of PB compared to ZB, the higher adsorption of DOC onto PB, or the promotion of aggregation, which might also explain the lack of DOC increases in PB-amended soils compared to controls [13,23,41] by a physical protection mechanism. This phenomenon is particularly notable at high biochar production temperatures [51]. The limited release from S + PB plots was also reflected by its differential DOC_{hw} $\delta^{13}C$ compared to S and S+ZB observed (Figure 4), resulting in limited capacity of DOC release and negative priming in S + PB. Moreover, the similarity of $\delta^{13}C$ of DOC_{hw} in S and S + ZB treatments was feasible, since in the last case most of the DOC_{hw} came from native organic matter due to positive priming.

Alternative explanations have been provided for priming effects, such as plausible CO_2-C release from carbonates that originated during or after biochar production [56], but this mechanism unlikely in our biochar due to its relatively low carbonate content (PB: 4.0 g kg^{-1}; ZB: 2.7 g kg^{-1}) and the neutral/basic pH of the soil that prevents rapid carbonate dissolution.

4.3. Persistence of Priming Effects in the Medium-Term

In the medium term (26 months after biochar application), a slight negative priming tendency was observed in both S + PB and S + ZB after a 30-day incubation (Figure 6). Many previous incubation investigations have indicated a considerable evolution in biochar-induced priming effects over time. This agrees with the expected long-term predominance of negative priming effects, as suggested in the meta-analysis [34]. This effect is probably due to the predominance processes the promote the of physical protection of organic matter by biochar (i.e., adsorption promoted by its porous structure) that cause a reduction in microbial activity. As an example, [28] observed that priming was suppressed during later incubation stages in soils amended with a grass type biochar. In this study, a negative priming effect following the application of hardwood-based biochar to soil was reported during the later stages of incubation (250 to 500 days). This might explain why the initial strong positive priming caused by ZB disappeared in the medium term (Figure 6). The minimal amount of organic carbon mineralized observed after incubating the medium-term samples support the hypothesis regarding the reduced availability of labile organic carbon, resulting from the low carbon inputs during the two years of the field test, due to the suppression promoted by organic fertilization during the experiment and the limited inputs from wine plant leaves, branches, and roots, as shown by the strong reduction in CO_2-C between field sampling times (Figure 1). Moreover, another mechanism underlying the negative PE revolves around the formation of soil aggregates, which shields SOC from microbial decomposition by enhancing organo-mineral interactions [57]. Small soil aggregates (<53 µm) containing SOC tend to exhibit greater resistance to agricultural practices compared to larger particles [58]. Furthermore, biochar amendments can enhance the physical protection of native SOC through induced soil aggregation [59], improving soil structure and soil health. Additionally, the adsorption of dissolved organic carbon (DOC) onto the BC surface leads to low decomposition rates and heightened stability. In this mechanism, the suppression of soil mineralization predominantly occurs during the later stages of incubation through two common processes: the encapsulation of organic matter (OM) within the BC pores and the sorption-mediated protection of OM on the BC surfaces. In contrast, DOC did not exhibit significant changes (Figure 2), and this observation, as

indicated in Figure 3, could be explained by a succession of microbial communities able to use carbon sources other than DOC during the experimental period and the reduced use of DOC. Therefore, the type of biochar may induce changes in microbial community composition, resulting in a transition from Gram-positive to Gram-negative bacteria, or vice versa [51]. Additionally, shifts in the ratio of soil bacteria to fungi can also occur [60]. The available carbon source determines the composition of the microorganism community. For instance, Gram-positive bacteria tend to prefer carbon derived from biochar, actively participating in the decomposition of aromatic carbon [61]. Conversely, the proliferation of Gram-negative bacteria is stimulated in the presence of readily degradable organic substances, such as dissolved carbohydrates, amino acids, and small polypeptides [61].

In addition to the change in priming tendencies observed between samplings and the increase in the relative biochar C contribution to the CO_2 released, the amount of degraded biochar was very low. This was observed despite the higher relative values in the medium-term sampling than that observed in the short-term sampling for both biochars (from 18% to 36% in S + PB and 17% to 77% in S + ZB).

Therefore, similar to the results described [19], we found that the decomposition rate of biochar decreased as the experiment progressed given that the biochar decomposition rate in the medium-term incubation test was less than 0.1 g kg^{-1} of biochar day^{-1} in all cases.

4.4. Isotopic Signature Differential Evolution during the 250-Day Incubations

For both sampling times, a trend of increased $\delta^{13}C$ was observed in the 30-day vs. the 250-day incubations in S and S + ZB samples (Figure 5) that could be attributed to a two-stage process. First, the selective preferential use of ^{12}C resulting from microbial metabolism together with ^{13}C enrichment in microbial biomass results in an initially high $\delta^{13}C$ in C-CO_2. After this and as the ^{12}C source is exhausted, this dying microbial biomass was decomposed, increasing the $\delta^{13}C$ of the evolved CO_2 [62]. For this reason, the $\delta^{13}C$ in the incubation air increases over time (given that limited fresh organic matter inputs are present in the plots). Conversely, the opposite $\delta^{13}C$ tendency was observed in S + PB samples, for which partial PB degradation could explain the trend for the decreased $\delta^{13}C$ of S + PB due to the natural ^{12}C richness of this biochar (Figure 5). However, such trends are not supported statistically since no significant differences in isotopic signature values were detected between treatments. This is probably related to the fact that (i) a simple two-compartment model was applied, i.e., only biochar and whole native soil organic matter isotopic ratios were taken into account without considering that other organic components could be affecting this measure (as fresh shoots, leaves, and microbial biomass); (ii) the evolved CO_2-C represents a very small portion of C of the whole SOM pool. Therefore, in future research or practical applications in agricultural settings, a more detailed study including the contribution of other present organic carbon sources and considering higher sample volumes with more representative CO_2-C emissions will allow these trends to be statistically validated.

5. Conclusions

- Priming effects were observed in a Mediterranean agricultural soil amended with a pine wood and a corn cob biochars, with clear and contrasted short-term effects depending on the biochar applied and with a strong attenuation of such effects to slightly negative priming after two years.
- In the short-term, the distinct priming effects of the two biochars depended on the feedstock and pyrolysis temperature used for its production. Wood biochars produced at high temperatures exhibited negative priming, while grass biochars produced at low temperatures manifested positive priming.
- The short-term positive priming is hypothesized to be driven by the highest labile organic carbon content in ZB biochar compared to PB.
- In the medium-term experiments, the strong initial priming effects were strongly attenuated, transitioning to slight negative priming in both biochars. This outcome

was anticipated after the depletion of the more labile carbon fraction in the more labile biochar and the promotion of physical protection processes by biochar that prevent priming.

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