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Pattern changes of Carbon-cycling enzyme activities as influenced by different C and N availability of organic materials

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ABSTRACT: The incubation study was conducted to investigate temporal pattern of changes along decomposition time of C-cycling enzyme activities in response to different chemical compositions of organic materials, particularly concentrations of C and N in residues applied. The experiment was divided into 4 treatments: 1) control (untreated soil), 2) soil + rice straw, 3) soil + biochar compost, and 4) soil + biochar. Experimental design was Randomized Complete Block design with 3 replications. The peak CO_2-C production at day 0 (3 h) of incubation was the highest in the rice straw treated-soil (36.1 mg CO_2-C kg/day) followed by biochar (33.1 mg CO_2-C kg/day) and biochar compost (32.2 mg CO_2-C kg/day). The highest cumulative CO_2-C production (0.78 mg CO_2-C /kg soil) was also observed in the soil + rice straw treatment. Increases in oxidizable organic C were positively correlated with CO_2 losses (r = 0.6656***), while microbial biomass C was also governed by CO_2-C production (r = 0.6553***). During the first 21 days of incubation, oxidizable organic C showed negative correlation with specific phenoloxidase (r = -0.7566***) and peroxidase (r = -05203**). Throughout the decomposition period, specific B-glucosidase activity showed positive correlation with N contents of organic materials both the initial to middle (r = 0.5673***), and the middle to later (r = 0.7653***) stages of decomposition. In addition, during the later period of decomposition, a positive correlation between total N and specific phenoloxidase activity was also observed. This study showed that C compounds degrading enzymes were influenced by C and N availability of organic materials in which the prominent parameter is N content. Our study also supported the positive role of N in regulating specific activity in degrading recalcitrant C compounds.

Keywords: C-cycling enzymes, microbial activities, organic materials, decomposition

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Introduction

Carbon -cycling enzymes have been extensively investigated for their expression of stimulated activities, particularly as influenced by N contents of organic materials which determine availability and quality of microbial substrates (Saiya-Cork et al. 2002; Sinsabaugh et al. 2005). Although it has been reported that application of contrasting quality organic materials can influence soil microbial community in relation to enzyme activities under long-term application (Miller et al. 2012; Kamolmanit et al. 2013), the pattern of changes of C-cycling enzymes along with decomposition time is still unclear. The temporal changes of enzyme dynamics might be influenced by N availability, but also by protection mechanisms of labile C in organic materials. In soil microbiological studies, the activities of hydrolases and glycosidases (e.g., invertase and B-glucosidase) coupled with their oxidative counterparts (e.g., phenoloxidase and peroxidase) have received much attention in soil quality (Moscatelli et al., 2012; Nannipieri et al., 2012; Armas-Herrera et al., 2013; Wang et al., 2013). The activities of easily-decomposable C compound-degrading enzymes were shown to be positively related to N concentrations in organic materials (Lucas et al., 2007; Aciego Pietri and Brookes, 2009; Bissett et al., 2011). As a consequence, the decomposition of C compounds is stimulated by N through enhancing activities of C-cycling enzymes (Kamolmanit et al., 2013). However, for resistant C compounds, the N effect on their degrading enzymes is still inconsistent. For example, the work of Carreiro et al. (2000) showed that addition of N had strong influence on inhibition of phenoloxidase activity in litter with high lignin concentration, but the opposite was true for decomposition of those containing labile C compounds. They also provided the first evidence that repression of oxidative enzyme activities during decomposition could be an important mechanism explaining the negative effects of N addition. On the other hand, Nitrogen has been reported to have positive effects on oxidative enzyme activities. This may result in depletion of soil organic matter (SOM) leading to priming phenomenon (decline of indigenous SOM) (Kuzyakov 2010). This is particularly true for phenoloxidase and peroxidase activities which still belong to a common oxidative group of enzyme involving in SOM dynamics (Sinsabaugh 2010). In these studies variation pertaining to concentrations of C and N in organic materials have been highlighted as important variables influencing the effects of N on soil enzyme activities (Carreiro et al. 2000; Keeler et al. 2009). Since C-cycling enzyme activities play an important role in residue decomposition, therefore, we chose oxidizable organic C as a comparative factor since it is a major component of soil functions, undoubtedly, in a low fertility soil.

Generally, biochar is very stable in soil compared to other organic materials, making its application to soils a suitable approach for soil C sequestration (Kuzyakov et al., 2009; Zimmerman et al., 2011). Previous studies showed that biochar produced under low temperature conditions contributed to N immobilization and microbial activity reduction (Deenik et al., 2010).

It was particularly evident that the production of biochar compost could reduce loss of N during composting (Liu et al., 2017) as well as increase some C-cycling enzyme activities of the final product (Kamolmanit et al. 2018). The increase in N during the composting process might be due to the activity of N-fixing bacteria which was expected to exist in the manure-based compost. As mentioned, biochar compost appeared to lead to higher soil organic C build-up than those of low-N organic materials. However, information on decomposition time during organic material degradation that N exerts influence on expression of C-cycling enzyme activities is still lacking. In addition, pattern changes of enzyme activities with time does not cover the whole decomposition cycle. This is especially so during the very early stage of decomposition. There has been a lot of work on the effects of indigenous soil N on C-cycling enzyme activities, however they did not distinguish sources of freshly applied N sources. Furthermore, as N is the key determinant of microbial decomposition of organic materials, it will be important to investigate how metabolic activities of soil microorganisms respond to the differentiation of N sources, particularly in sandy soils where original N concentrations are very low (Kamolmanit et al., 2013). In this regard, in-depth investigations on temporal changes of enzyme activity during microbial decomposition of organic materials as influenced by N from fresh residues may provide an insight into mechanisms, (e.g., N changes some microbial activities, or N directly influences the enzymes) that N induces the changes in expression of C-cycling enzyme activities. We hypothesized that the activities of enzyme in soils is controlled by concentrations of C and N availability. The objectives of this study were to: 1) investigate the decomposition patterns of organic materials through measurement of the activities of C-cycling enzymes degrading labile- (e.g., invertase and B-glucosidase) and resistant- (e.g., phenoloxidase and peroxidase) C compounds; 2) investigate the response of those activities of enzymes to contrasting freshly applied N sources during different stages of decomposition; and 3) use of microbiological parameters to evaluate changes in soil organic C dynamics and accumulation.

Materials and methods

Soils and organic materials used

Soil used in this study was obtained from a field experiment at the Agricultural Training and Research Center, Nakhon Ratchasima Rajabhat University, Thailand. The soils were air dried, sieved through 2 mm sieve, and maintained at room temperature until the start of incubation experiments. The top layer of the soil (0-15 cm) showed loamy sand texture (71.1 %, 12.6 %, and 16.3 %) and bulk density of 1.34 g/ cm3. The experiment had three parallel organic material-treated treatments with rice straw, biochar and biochar compost. Biochar was traditionally produced from the same feedstock of Siamese Neem Tree (*Azadirachta indica*), East Indian Walnut (*Samanea saman*), Ma Kha Num (*Sindora siamensis*), Burma Padauk (*Pterocarpus macrocarpus*), Teng (*Shorea obtusa*) and Rang (*Shorea*

siamensis). The temperature of Thai traditional kiln used for production of biochar was approximately 350 °C. Goat manure was obtained from the animal farm located at the Suranaree University of Technology, Nakhon Ratchasima, Thailand. Biochar compost was prepared from the mixture of rice straw and biochar at the ratio of 1: 1 coupled with goat manure at an initial C to N ratio of 30: 1 (w/w) seeded with a microbial activator at a ratio of 50 g: 1000 g of residue mixture. Preparation and some properties of biochar composts can be found in Kamolmanit et al. (2018). For incubation study, organic materials (i.e., rice straw, biochar, and biochar compost) were air-dried and chopped into smaller pieces using a blender, ground and passed through a 2 mm sieve. Subsamples were then stored and for further analysis and usage. The analytical characteristics of the organic materials and soils are showed in **Table 1**.

Experimental set-up and maintenance

The incubation experiment was conducted in an incubation room under non-leaching conditions during June to August 2018. The experiment has 4 treatments organized in randomized complete block (RCB) design with three replications. The experiment was divided into 4 treatments include the following: 1) control (untreated soil), 2) soil + rice straw, 3) soil + biochar compost, and 4) soil + biochar. One kilograms of air-dried soils were put in 1 litre plastic bottles (approximately 7.5 cm diameter and 18 cm height). Organic materials were applied to soil at the rate of 1 g C / kg soil (dry weight). Organic materials pertaining to each treatment was thoroughly mixed with soil. Initial soil water of each microcosm was adjusted to 60% water holding capacity with distilled water based on 13% (w/w) field capacity water content under the soil used in this study. During the incubation, experimental units were opened to allow air exchange, kept at 25 °C and watered to keep constant moisture. Soil samplings were done at the interval of 0, 3, 7, 21, 42 and 63 days after incubation. The interval designated 0 was the sampling done 3 h after the start of the incubation experiments. The experimental unit was taken to represent each treatment at each sampling time. Immediately after soil sampling, the remaining weeds was removed from the soil. The soil samples were kept at -20 °C until further analyses.

Soil analyses

Soil pH was determined in a 1: 2.5 (soil: H_2O) using a pH meter. Oxidizable organic C was measured using $K_2Cr_2O_7$ oxidation and subsequent determination of the remaining dichromate by titration with ferrous sulfate (Black, 1965). Total N was determined using the micro-kjeldahl method. Soil microbial biomass C and N were measured by the chloroform fumigation–extraction method (Amato and Ladd, 1988). For microbial biomass C, twenty grams of soils were extracted with 100 ml of 0.5 M K_2SO_4. Microbial biomass C was determined after oxidation with $K_2Cr_2O_7$ and C content was the difference between the values of fumigated and unfumigated samples, using a KEC factor of 0.33 (Sparling and West, 1988). Microbial biomass N was determined by the chloroform fumigation-extraction technique (Amato and Ladd, 1988). Calculation of microbial

biomass N was performed employing a KEN factor of 3.1 (Amato and Ladd, 1988). CO_2-C production was determined using alkaline trapped method. A glass bottle containing 20-25 ml of 1 N NaOH to trap the CO_2 evolved was placed by hanging in the bottles. Trapped CO_2 was determined by titration the excess alkaline with 0.5 M HCl. Specific activity expressed per unit of C in soil. Invertase was measured as described by Schinner and von Mersi (1990) using 50 mM sucrose as substrate. Specific activity of invertase was expressed as mg glucose equivalent (GE) g C /3 hour. B-glucosidase activity was measured as described by Alef and Nannipieri (1995) using 25 mM p-nitrophenyl-B-d-glucopyranoside as substrate. Specific activity of B-glucosidase was expressed as µg p-nitrophenol g C /hour. Phenoloxidase and peroxidase activities were measured as described by Hendel et al. (2005) using 5 mM L-3,4-dihydroxyphenylalanine as substrate. For peroxidase activity, 0.3% (v/v) hydrogen peroxide was used. Specific activity of phenoloxidase was calculated using 1.66 µmol as the extinction coefficient for L-DOPA, while specific activity of peroxidase was calculated as the difference in activity between samples treated with and without H_2O_2. Activities of phenoloxidase and peroxidase were expressed as µmol 2,3-dihydroindole-2-carboxylate (dicq) g C /hour.

Statistical analysis

One factor analysis of variance (ANOVA) under randomized complete block (RCB) design in conjunction with least significant differences (LSD) were used to analyze the main treatment effects on assayed soil parameters ($P < 0.05$) (Statistix 8.0, Analytical software 2003). Pearson's correlation analysis was conducted to study relationships between enzyme activities and other soil parameters.

Table 1 Initial properties of soil and organic materials used in the incubation study

Parameters	Soil	Organic materials			
		Goat manure	Rice straw	Biochar	Biochar compost
Moisture content (%)	6.2	10.4	9.6	5.9	24.8
pH[a]	5.4	7.98	5.9	10.5	7.96
EC (mS/m)[a]	0.18	8.02	0.41	2.24	3.89
CEC ($cmol_c$ /kg)	1.78	nd	nd	nd	nd
Ash content (%)	nd	24.2	17.7	2.21	11.5
Organic C (g C /kg)	1.58	254	385	100.4	190.3
Total N (g N /kg)	0.07	12.6	5.4	1.04	8.17
C to N ratio	22.6	20.2	71.3	96.5	23.3

nd: not determined. EC: electrical conductivity. CEC: cation exchange capacity. [a]soil: H_2O = 1: 2.5, organic materials: H_2O = 1: 10.

Results and discussion

CO_2-C production reflecting microbial decomposition activities

The effects of biochemical composition of C substrates on CO_2-C production can be observed in soils added with easily decomposable C compounds-containing residue like rice straw. The initial mean CO_2-C production of all organic material-treated treatments was approximately 33.8 mg CO_2-C /kg/day, and then gradually dropped to 9.82 mg CO_2-C /kg/day at day 21 **(Figure 1a)**. In this sense, decrease in CO_2-C production resulted from the oxidation of C to CO_2 by soil microorganisms. Our results are compatible with the findings of Ibrahim et al. (2015) who found that the CO_2 fluxes decreased significantly with the increase of incubation times in a paddy soil treated with rice straw at 60 % soil WHC. During first 21 day of incubation, the greatest CO_2-C production among the organic materials was under the rice straw (11.3 - 36.1 mg CO_2-C /kg/day) ($P < 0.05$), while the lowest was observed in the biochar material amendments (8.5 – 33.1 mg CO_2-C /kg/day). The production of CO_2 at the initial stages of decomposition were governed by the ratio of C to N of organic materials (r = 0.6656**) and microbial biomass C (r = 0.6653***) **(Table 2)**. Our results were in agreement with Lou et al. (2007) who found that soil CO_2 fluxes positively correlated with soil microbial biomass C. Meanwhile, Ibrahim et al. (2015) also noticed that rice straw incorporation into the paddy soil caused significant increases in CO_2 fluxes as compared to unamended paddy soil. The incorporation of rice straw into soil greatly increased CO_2 production because it played an important role as a source of readily C available for soil microorganisms (Lou et al., 2007). These findings support the present study in rice straw amended loamy soil which had higher oxidizable organic C with significant increases in CO_2-C production. During the decomposition process, high CO_2 production reflected a low efficiency of C utilization by soil microbes, which was associated with their chemical compositions, particularly oxidizable C, N and resistant C contents. It is notable that not only N-rich organic material but also N- and lignin-poor rice straw which stimulated CO_2-C released as shown by the significantly highest respiration within first 7 day after fresh rice straw addition into soil. Puttaso et al. (2011a) explained that the stimulation of microbial respiration by rice straw application is because it served as relatively easily decomposable C compounds (e.g., cellulose) with readily degraded to CO_2. This is in agreement with our results which showed that highest CO_2-C production was observed under rice straw-treated soil. Cumulative CO_2-C production significantly increased ($P < 0.05$) with increasing rate with time in all organic material-treated soils. In the presence of organic materials, the maximum CO_2-C production of soil added with rice straw, biochar compost, and biochar were 815.7, 695.8 and 660.5 mg CO_2-C /kg soil, respectively.

Although CO_2-C production showed no significant differences among treatments with rice straw and biochar compost incorporations, the rice straw amendment led to significantly increased cumulative CO_2-C released as compared to the other treatments **(Figure 1b)**. The significantly higher cumulative respiration in soil added with fresh labile residues (e.g., rice straw) as compared to the two more resistant residues at all stages of decomposition, indicated that not only N compound, but also cellulose substrate was a key factor regulating microbial metabolisms. Our results showed that organic materials with high N but low cellulose content, like biochar compost, were most effective among treatments in accumulating soil microbial biomass C through reducing microbial CO_2-C production. This is because incorporation of easily decomposable C compound like fresh rice straw into soil can enhance the immobilization of N and increases CO_2 released in soil due to high C/N ratio (Hossain, 2018). Thus, in our case, the oxidizable organic C was positively correlated with CO_2-C production (r = 0.5333***) suggesting that readily available C compound of organic materials was required and could be utilized as an energy source for microbial growth.

Table 2 Pearson correlation coefficients between microbial activities and soil organic C, total N and microbial biomass C and N after organic materials incubation.

Parameters	Period (day)	CO_2 Production	Specific enzyme activities			
			Inv	B-glu	Phenolox	Perox
Organic C	0-21	0.6656***	0.4044	0.2840	-0.7566***	-0.5203**
	42-63	0.2785	-0.4403	-0.2702	-0.4046	-0.1813
Total N	0-21	-0.2803	-0.1498	0.5673***	0.0436	0.2545
	42-63	0.2835	0.6176***	0.7653***	0.5595***	0.3658
Microbial biomass C	0-21	0.6653***	-0.4215	-0.0339	-0.4061	-0.2532
	42-63	0.4775	0.1745	0.0479	0.0052	-0.0352
Microbial biomass N	0-21	-0.1690	-0.0469	0.3682	0.0159	0.3023
	42-63	0.1013	0.5450***	-0.0448	0.2275	-0.0542

, * Significantly different at $P < 0.01$ and 0.001, respectively. Inv = Invertase, B-glu = B-glucosidase, Phenolox = phenoloxidase, Perox = peroxidase. Specific activities are presented as potential activity per C unit.

Temporal changes of enzyme activities as influenced by availability of C and N

Easily decomposable C compound-degrading enzymes

Throughout the middle to later of decomposition process, specific invertase activity in soil + biochar compost treatment was higher than those of the other treatments ($P<0.05$). The specific invertase activities of soil + biochar compost treatment showed highest activities at day 21, 42 and 63 (0.54, 1.24 and 1.34 mg GE /g C /3hour, respectively) (**Figure 2a**). Kamolmanit et al. (2018) found that rice straw incorporation into biochar compost promoted enzymes degrading labile C compounds, such as invertase and B-glucosidase activities. Moreover, Kamolmanit et al. (2013) also explained that rice straw decomposition produced cellulose and some residual sugars which was the key substrate for microbial growth, and maintained higher enzyme production. Meanwhile, composting is a biological decomposition process, wherein resistant C compounds of organic materials is degraded to achieve available nutrients at the end. As a consequence, N in biochar compost was more accessible to microbes than biochar. Moreover, specific invertase activity in soil added with biochar compost was higher than those of the other treatments might also be due to a larger amount of N supplied in biochar compost, specifically, large amount of goat manure was added into the compost mixture prior to the composting process. Not only N that exerted stimulation effects on invertase activities, but also did easily decomposable C compounds. This is shown by contrasting patterns of invertase activities between two groups of contrasting chemical composition organic materials (i.e., rice straw and biochar vs. biochar compost) **(Figure 2a)**. Decreases in specific invertase under high cellulose-containing organic materials group were likely due to exhaustion both of available residual sugars and N, while increases in the activities under biochar compost treatment may have been due to availability of N. Specific activities B-glucosidase showed different responses to organic material addition, but a significant difference ($P<0.05$) was only found at day 7, 42 and 63 **(Figure 2b)**. The specific B-glucosidase activity was sharply increased in biochar-treated soil (196.75 µg p-nitrophenol /g C /hour) as compared to control (71.9 µg p-nitrophenol /g C /hour) during the first 7 days of incubation (**Figure 2b**). Meanwhile, the specific B-glucosidase activity in untreated soil was lower than those of the other treatments at day 7 until the end of decomposition. In contrast to invertase, specific B-glucosidase activity in biochar compost-treated soil was lowest among all organic material-treated treatments after day 7. This was explained by the limited availability of C due to low cellulose concentrations in biochar compost product as previously reported by Kamolmanit et al. (2018). Interestingly, biochar gave as high or higher specific B-glucosidase activities than rice straw-treated soils. This clearly substantiates the critical role of N availability in stimulating the activity of labile compound degrading enzymes such as B-glucosidase. Support for this assumption was provided in soil treated with biochar, in which B-glucosidase activity was higher than

the other treatments (day 7, 42, and 63). Interestingly, when fresh biochar was added into soils, the new activity stimulation took place after day 21 which was likely arisen from availability of previously-protected cellulose in the biochar. One mechanism was referred to complexation of cellulose by lignin in plant cell walls (Talbot and Treseder 2012). This suggested that not only the availability of C, but also different protection mechanisms against cellulose decomposition influenced the activity of B-glucosidase. Additionally, although rice straw has the highest oxidizable organic C (385 g/kg) among the organic materials studied, it did not translate into stimulation of specific B-glucosidase activity. This can be seen in significantly lower specific B-glucosidase activities at than biochar **(Figure 2b)**. Our study pointed out to the significant role of biochemical interactions between different compounds, as shown in this case by that of cellulose, lignin, and N constituents of organic materials in regulating microbial decomposition, as manifested in invertase and B-glucosidase activities, of organic materials. The low N concentration of rice straw led to overall low N environment which did not stimulate early increase of B-glucosidase activity and the stimulation was delayed until the middle stages of decomposition.

Resistant C compound-degrading enzymes

Specific phenoloxidase activity was significant only in soil + biochar treatment at day 42 and 63 (3.6 and 2.6 µmol dicq /g C /hour, respectively) ($P<0.05$) (**Figure 2c**). Meanwhile, highest specific peroxidase activity was observed in soil + rice straw treatment at day 42 (2.44 µmol dicq /g C /hour) ($P<0.05$) **(Figure 2d)**. Sharp increase in phenoloxidase and peroxidase activities (day 21-63) in organic material-treated treatments at

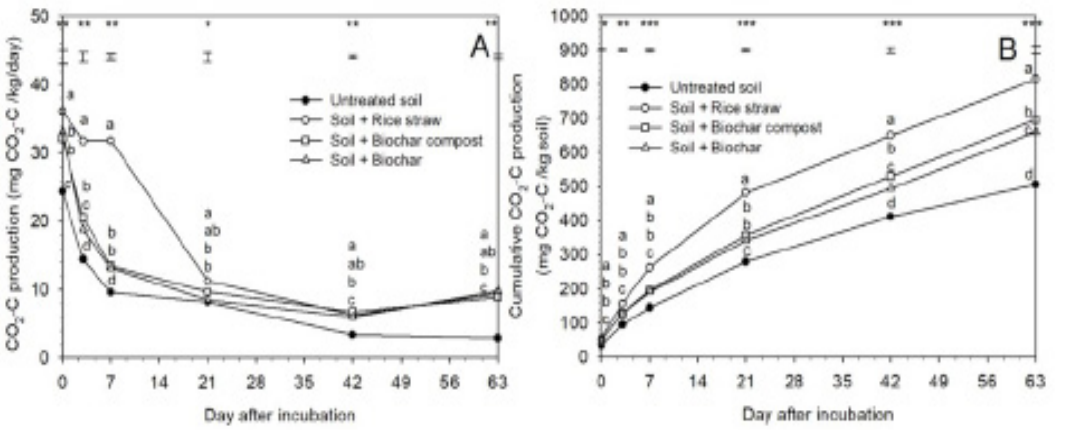

Figure 1 Dynamics of CO_2-C production (A) and its accumulation (B) as affected by contrasting chemical compositions of organic materials. Vertical bars represent standard error of the difference (SED). Different letters indicate significant difference at $P<0.05$ (LSD).

middle to later stages of decomposition indicated adjustments of soil microbes to resistant C substrates of all inputs. This is because lignin has been found to be another important factor parameter regulating organic residue decomposition (Hadas et al., 2004; Rubin, 2008). Although, N was found to have profound effects on stimulating expression of enzyme degrading labile C compounds at all stages, it regulate the expression of enzymes degrading resistant C only at the later stage of decomposition. In this study, at least, we observed no suppressing effect of N availability on specific phenoloxidase and peroxidase activities. This was evident from the increases of specific phenoloxidase activity in soils treated with N-rich organic materials (e.g., biochar compost). As support for this, during day 42–63, the specific phenoloxidase activity was highly positively correlated with total N ($r = 0.5595^{***}$) indicating that peroxidase was not affected by N, while it is likely that both C and N sources had stimulation on phenoloxidase activity **(Table 2)**. This assumption was supported by Keeler et al. (2009) who found no effect from long-term organic N inputs on lignin-degrading enzymes in soils under forest and grassland. On the other hand, our findings do not support the ongoing discussion on the inhibitory effect of N on the activity of lignin-degrading enzyme (Berg and Matzner, 1997). Even though some studies have reported that concentration of N in organic inputs and/or in soils have negative effects on activities of this enzyme, our results showed that there was positive or at least no negative effects of N on phenoloxidase activity. Notably, a rapid activity increase of phenoloxidase in soils added with biochar and biochar compost after day 21 was rapidly increased. Such stimulation was confirmed by Kamolmanit et al. (2013) who found that Myceliopthora thermophila and Anguillospora longissima, two fungi with known phenoloxidase activities, were particularly stimulated in the decomposition of low-N leaf litter. In the present study, when the low N condition existed, particularly under a low fertility sandy loam soil, substrate deprivation resulted. This led to microbial adaptation of substrate use from labile to more resistant C compounds.

Conclusions

C-cycling enzyme activities were affected by chemical compositions of organic materials in the short term of incubation. Although, C compounds constituents of organic materials exerted stimulating effects on activities of enzymes degrading resistant C compounds they did not affect those degrading labile C compounds. Nitrogen regulate the expression of enzymes degrading labile C compounds at all stages, while, this source of N regulate that of enzymes degrading resistant C only at the later stage of decomposition. For activities of enzymes degrading labile C compounds, N from organic material sources exerted significant influence on both of these enzymes (invertase and B-glucosidase), however only N-rich material, like biochar compost, that demonstrated the influence middle to later in the decomposition period. Meanwhile, in N-poor residue, notably biochar, became dominant over N compounds in stimulating activities of B-glucosidase

during the same decomposition period. In contrast to enzymes degrading labile C compounds, degrading resistant C compounds, notably peroxidase, were not affected by N. However, only phenoloxidase, but not peroxidase, was stimulated by N at the later stage of decomposition. As expected, this study suggests that not only available C but also N contents coupled with recalcitrant C compounds of organic materials leading to an enhanced microbial utilization of C and N in organic materials. Therefore, we pointed out that biochar compost are most suited to improve soil organic C content in a low fertility loamy sand soil. Moreover, it can be expected that in the long-term addition of biochar compost will be mineralized for plants and soil microbes more quickly than biochar or rice straw alone.

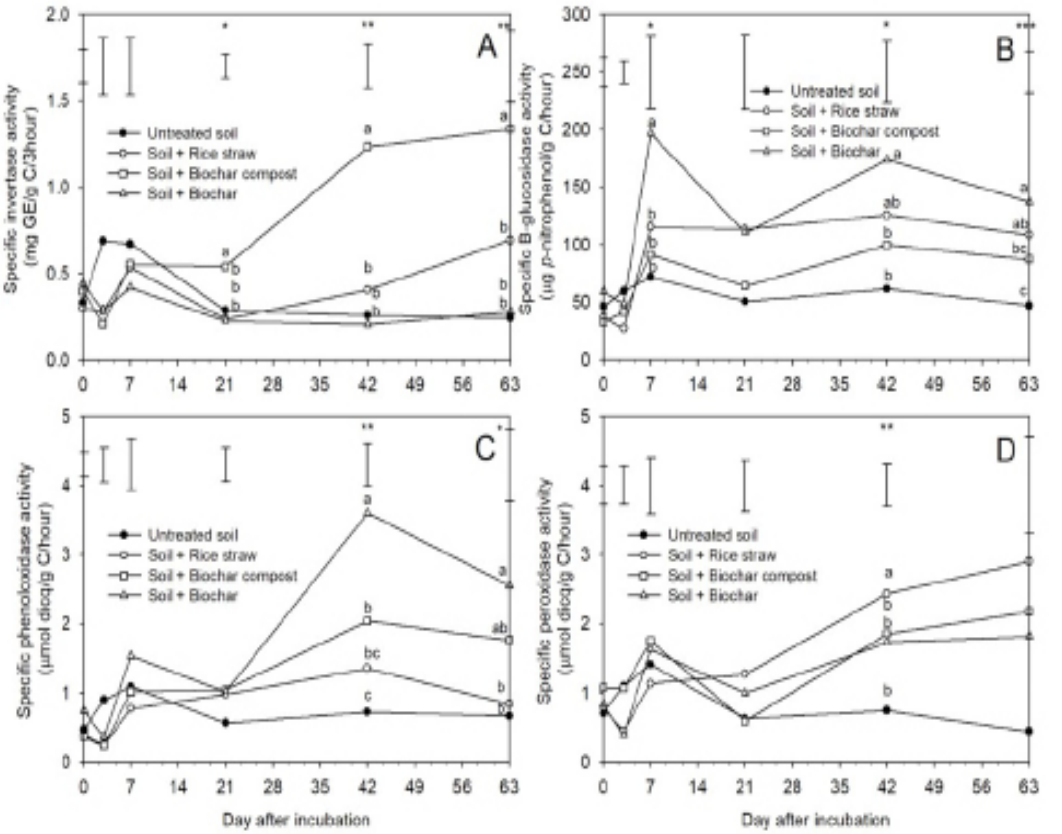

Figure 2 Specific activities of invertase (A), B-glucosidase (B), phenoloxidase (C), and peroxidase (D) in soil treated with different organic materials. Vertical bars represent standard error of the difference (SED). Different letters indicate significant difference at $P < 0.05$ (LSD).

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