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Influence of Various Biochars on the Survival, Growth, and Oxidative DNA Damage in the Earthworm *Eisenia Fetida*

Won-Il Kim,¹ Anitha Kunhikrishnan,¹ Woo-Ri Go,¹ Seon-Hee Jeong,¹ Gyeong-Jin Kim,¹
Seul Lee,¹ Ji-Hyock Yoo,¹ Namjun Cho¹ and Ji-Ho Lee^{2*}

¹Chemical Safety Division, National Academy of Agricultural Science
Rural Development Administration, Jeonju, 565-851, Korea

²Accident Prevention and Assessment Division, National Institute of Chemical Safety, Daejeon, 305-343, Korea

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Abstract

BACKGROUND: Biochar (BC) has a great potential for enhancing soil fertility and carbon sequestration while facilitating beneficial waste disposition. Therefore, it is essential to assess and mitigate any inadvertent consequences associated with soil biochar amendment. Earthworm activity is very vital in the soil system, yet there are a limited number of studies that have examined their impact resulting from biochar application to soil.

METHODS AND RESULTS: In this study, the survival, growth, reproductive tests, and oxidative DNA damage tests (measured by 8-hydroxydeoxyguanosine (8-OHdG) and catalase (CAT) activities) to assess the potential toxicity to earthworm *Eisenia fetida* in artificial soil amended with BCs were investigated. The BCs derived from perilla meal, sesame meal, and pumpkin seed were pyrolyzed at 300 and 550°C, and then amended with soil at a rate of 5%. All the earthworms survived, but lost weight compared to control soil after 28 day incubation period. Moreover, the BC-amended soils did not significantly affect the cocoon numbers of earthworms. Slightly higher

concentrations of 8-OHdG and CAT were observed in earthworms present in BC-treated soil than those in control soil. Furthermore, the 8-OHdG concentrations in the soil amended with BC produced at 550°C were greater than those at 300°C, and it slightly decreased as the incubation time increased.

CONCLUSION: These observations could be due to higher contents of toxic metal(loid)s and also higher pH in BCs pyrolyzed at 550°C than 300°C. While BC is efficiently being used in agricultural fields, this study suggests that it is required to assess the unintended negative impacts of BC on soil ecosystems.

Key words: Biochar, Earthworm, Oxidative damage, Perilla meal, Pumpkin seed, Sesame meal

Introduction

Biochars (BCs) are products of thermal degradation of organic materials by pyrolysis (Lehmann and Joseph, 2009). The BCs derived from agricultural biomass wastes are increasingly regarded as beneficial materials for soil and environmental remediation (Lehmann *et al.*, 2011). The BC-amended soil can improve soil fertility for crop production, carbon

*교신저자(corresponding author): Ji-Ho Lee
Phone: +82-10-2268-5513; Fax: 042-605-7065;
E-mail: jhlee76@korea.kr

sequestration for mitigating the green house gas, and removal of various pollutants (Lehmann *et al.*, 2006). Also, the BCs have been also shown to change the composition and abundance of biological community in soil (O'Neill *et al.*, 2009). These effects depend on the BC feedstock and pyrolysis conditions (Chan *et al.*, 2009; Gaskin *et al.*, 2008) as well on the soil itself (Speir, 2008). Among the agricultural biomasses, the total biomasses of perilla, sesame, and pumpkin crops are amounted to be 167,427, 117,563, and 300,000 ton/year, respectively, in Korea (Hong *et al.*, 2004).

Earthworms have been considered as the most representative soil fauna worldwide, since they perform many essential and beneficial functions in soil ecosystems, including decomposition, nutrient mineralization, and soil structure improvement (Edwards and Bohlen, 1996). Their ability to perform these functions can be inhibited upon exposure to harmful substances. Many ecotoxicological studies have used earthworms as a model system to assess the potentially toxic materials in soils (Amorim *et al.*, 2005; He *et al.*, 2007; Lanno *et al.*, 2004; Lukkari and Haimi, 2005; Zhou *et al.*, 2007). Earthworms are useful model organisms because their response to environmental disturbances can be assessed and related to environmental outcomes, including their avoidance behavior, enzyme activity level, growth rate, mortality, and reproduction patterns (Yeardley *et al.*, 1996).

Several studies have focused on the beneficial effects of BCs on the crop growth, reduction of greenhouse gas emission, and removal of toxic compounds (Woolf *et al.*, 2010; Carter *et al.*, 2013). Nevertheless, the harmful materials released from pyrolyzed BC unintentionally may inhibit the soil organisms (Lehmann *et al.*, 2011; Weyers and Spokas, 2011). Pyrolyzed BCs contain toxic metal(loid)s, PAHs, and dioxins with various isomers, which are associated with oxidative stress through accumulation of reactive oxygen stress (ROS) in cellular tissues (Stegeman and Lech, 1991; Lopez *et al.*, 2006). Particularly, the toxic metal(loid)s are involved in accumulation of ROS caused by imbalance between oxidants and antioxidants during metabolism, thereby inducing cell toxicity as well as potential carcinogenesis (Shi *et al.*, 2004). Malondialdehyde (MDA) as a biomarker of oxidative stress is an oxidation product generated by the interaction between ROS and polyunsaturated fatty acids (Song *et al.*, 2012). There is, however, a lack of agreement whether various BCs lead to negative or

positive impact on the potential toxicity of earthworms. Furthermore, only very few researchers have studied the potential impact of BCs in soil on the toxicity and oxidative damage in earthworm tissues.

Previous studies have shown different response of earthworms to biochar in soil. In some cases, earthworms exhibit a preference for soils amended with biochar (Chan *et al.*, 2008); the liming potential of the biochars was suggested as a factor that influences this preference (Topoliantz and Ponge, 2005; Topoliantz *et al.*, 2005; Liesch *et al.*, 2010). Not all biochars are, however, preferred by earthworms; toxicity of biochar compounds to earthworms (Liesch *et al.*, 2010) and insufficient moisture (Li *et al.*, 2011) are other factors that influence earthworm preferences for certain biochar-amended soils. Also, both the application rate of biochar and the original feedstock used have been shown to affect the soil biota. Weyers *et al.* (2009) reported that application rates higher than 67 t ha⁻¹ of biochar made from poultry litter had a negative impact on earthworm survival rates. They hypothesized that increased soil pH or salt levels may have been the reason for the observed reduced survival rates. Liesch *et al.* (2010) noted that earthworm activity was greater in soil amended with pine chip biochar than with poultry litter biochar. Recent work by Van Zwieten *et al.* (2010) has shown that earthworms preferred biochar-amended ferrosols over control soils, although they found no significant difference for calcarosols. The earthworm reports cited above using biochar highlights the complex dynamic interactions which can vary greatly with soil type, application rate, feedstock used, and the presence of toxic materials.

In order to develop appropriate recommendations for biochar application, the potential impacts on soil biota, specifically earthworms, need to be identified. The present study was undertaken to evaluate the effect of various BCs, i.e. perilla and sesame meals, and pumpkin seeds on the survival, growth, reproduction, and oxidative stress, measured by catalase (CAT) and 8-hydroxydeoxyguanosine (8-OHdG) activities on the earthworms.

Materials and methods

Biochar preparation and characterization

The BCs were produced from perilla and sesame meals, and pumpkin seed at two different temperatures

(300 and 550°C) by modifying the procedure reported by Yang *et al.* (2010). Selected biomasses were air-dried, ground to pass through a less than 2 mm sieve, placed into porcelain crucibles with lids, and pyrolyzed for 3 h at 300 and 550°C under limited oxygen in a muffle furnace. The pH and electrical conductivity (EC) in pyrolyzed BCs were determined using end-over-end equilibration of soil with water at a ratio of 1:5 for an hour and measuring the solution with calibrated pH and conductivity meters (250A, Thermo Orion, Beverly, MA, USA). For proximate analysis, the contents of moisture, ash, mobile matter, and resident matter were measured by following the modified thermal analysis method suggested by McLaughlin *et al.* (2009). The moisture was determined by calculating the weight loss after heating the BCs at 105°C for 24 h to a constant weight. The ash contents were measured by heating the BCs in open-top crucibles at 700°C for 1 h. Mobile matter was determined as the weight loss after heating in a covered crucible at 450°C for 30 min. The resident matter was calculated by the difference in moisture, ash, and mobile matter. The contents of trace metal(loid)s in selected BCs were acid-digested with concentrated HNO₃ for 5 days, filtered (Hyundai Micro Co., Ltd No. 52, Seoul, Korea), diluted, and analyzed by ICP-OES (GBC Scientific Ltd., Australia) using the modified procedure described in US EPA method 3051A (US EPA, 1995).

Artificial soil preparation and incubation

Artificial soil was prepared by mixing 10% *Sphagnum* peat moss (previously sieved through 2 mm mesh), 20% kaolin clay, and 68% quartz sand as described by US EPA guideline (US EPA, 1996). 2% calcium carbonate was added to adjust the soil pH to 6.5 ± 0.5 , and moistened to 35% by weight with Milli-Q ultrapure water. The pyrolyzed BCs at two temperatures were amended with 300 g soil at an application rate of 5%, and incubated for a week to allow equilibration. All the treatments per experiment were performed in triplicates.

Earthworm toxicity test

Growth and reproductive tests

In this study, the earthworm *Eisenia fetida* was chosen, which is widely used as a model soil organism in research (Van Zwieten *et al.*, 2010; Li *et al.*, 2011). The earthworm subchronic toxicity test was conducted by following a modified protocol developed by US EPA

(US EPA, 1996). The earthworms were randomly selected in a range of 300-600 mg, and depurated on filter paper hydrated with ultrapure Milli-Q water for 24 h. Ten adult *E. fetida* earthworms were weighed and transferred to 500 mL glass jars, which contained the soil amended with BCs produced at 300 and 550°C at 5% application rate. These glass jars were covered in parafilm(with air holes) to prevent earthworm escape, and incubated at a temperature of $20 \pm 2^\circ\text{C}$, and relative humidity above 85% for 28 days in dark. The number of worms alive in each container was counted after 28 days. Growth was measured by weighing worms at the end of the exposure period, to determine the mean percentage growth of the population relative to the mean initial weight. Cocoons were collected at the end of the experiments by wet-sieving the soil from each container.

8-hydroxydeoxyguanosine (8-OHdG) and catalase (CAT) activity measurement

The earthworms exposed to BC-treated soil were picked out, and depurated on a hydrated filter paper (Whatman®, Brentford, UK). The earthworm tissue was homogenized by a moto-homogenizer (ART-MICCRA D-8, Mullheim, Germany) on ice with a cold buffer (i.e. 100 mM potassium phosphate, pH 7.0, 2 mM EDTA) for CAT activity. For the measurement of 8-OHdG as an indicator of oxidative DNA damage, the earthworm was also homogenized with cell extraction buffer (Invitrogen, Camarillo, CA, USA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Amresco, Cochran Solon, OH, USA). The homogenized samples were centrifuged at 13,000 rpm for 15 min, and the supernatants were taken for CAT and 8-OHdG measurements at 0, 10, 20 days. The CAT activity was assayed by measuring the formaldehyde produced by the reaction of methanol with enzyme in the presence of H₂O₂. The amount of formaldehyde was determined using purpald as a chromogen at 595 nm. One unit of CAT was defined as the amount of enzyme to cause the formation of 1 nmol formaldehyde per min at 25°C. Oxidative DNA damage test was performed using OxiSelect™ Oxidative DNA damage ELISA Kit (Cellbiolabs, San Diego, CA, USA). Briefly, the supernatants were added to an 8-OHdG/BSA conjugate pre-absorbed EIA plate. After a brief incubation for 10 minutes, an anti-8-OHdG monoclonal antibody was added, followed by an HRP conjugated secondary

Table 1. The pH, electrical conductivity, and proximate analysis of various biochars derived from perilla, and sesame meals, and pumpkin seed at 300 and 550 °C

Biochars	pH (1:5)	pH (1:5) ^a	EC (1:5) (mS/cm)	EC (1:5) ^a (mS/cm)	Yield (%)	Moisture (%)	Ash (%)	Mobile matter (%)	Resident matter (%)
Perilla meal-300	8.80 ± 0.03	7.67 ± 0.13	0.22 ± 0.01	0.23 ± 0.07	58 ± 0.7	0.86 ± 0.03	13.50 ± 0.2	58.50 ± 1.2	27.10 ± 0.5
Perilla meal-550	10.30 ± 0.10	7.96 ± 0.70	0.55 ± 0.05	0.22 ± 0.20	33 ± 0.1	1.03 ± 0.05	19.6 ± 0.3	47.50 ± 1.0	31.90 ± 0.2
Sesame meal-300	8.63 ± 0.03	7.58 ± 0.72	0.41 ± 0.02	0.30 ± 0.20	62 ± 0.5	1.52 ± 0.02	6.50 ± 0.4	56.00 ± 0.8	36.00 ± 0.5
Sesame meal-550	10.12 ± 0.05	8.18 ± 0.51	1.01 ± 0.08	0.43 ± 1.34	34 ± 0.7	0.50 ± 0.01	11.70 ± 0.1	42.50 ± 0.7	45.30 ± 0.2
Pumpkin seed-300	7.64 ± 0.08	7.18 ± 0.43	0.28 ± 0.06	0.59 ± 0.40	64 ± 0.1	1.09 ± 0.08	5.50 ± 0.2	68.30 ± 0.7	25.10 ± 0.1
Pumpkin seed-550	7.81 ± 0.07	7.68 ± 0.76	1.06 ± 0.10	0.37 ± 0.81	26 ± 0.7	0.72 ± 0.10	10.10 ± 0.1	41.30 ± 0.6	47.90 ± 0.8

^a Measured values in artificial soil amended with 5% biochar; Measured values are expressed as mean ± SD of triplicates; The pH and EC values in control soil were 7.03 ± 0.03 and 0.18 ± 0.08 mS/cm, respectively.

Table 2. The contents of trace metal(loid)s in biochars derived from perilla, and sesame meals, and pumpkin seed at 300 and 550 °C

Biochars	As (mg/kg)	Cd (mg/kg)	Cr (mg/kg)	Cu (mg/kg)	Ni (mg/kg)	Pb (mg/kg)	Zn (mg/kg)
Perilla meal-300	ND	0.05 ± 0.07	4.11 ± 0.01	70.56 ± 3.43	7.69 ± 0.16	20.33 ± 0.17	163.04 ± 14.53
Perilla meal-550	ND	1.86 ± 0.02	12.50 ± 0.05	131.60 ± 2.20	8.46 ± 0.11	20.61 ± 0.05	373.24 ± 10.30
Sesame meal-300	ND	0.39 ± 0.01	4.20 ± 0.36	60.32 ± 1.22	8.94 ± 0.11	16.83 ± 4.60	149.92 ± 17.52
Sesame meal-550	ND	2.77 ± 0.22	6.74 ± 0.69	103.84 ± 17.51	7.17 ± 1.56	16.14 ± 3.86	316.15 ± 90.23
Pumpkin seed-300	ND	ND	3.01 ± 1.98	86.62 ± 58.33	23.87 ± 6.10	17.04 ± 4.27	181.26 ± 11.86
Pumpkin seed-550	ND	ND	9.65 ± 2.07	91.20 ± 2.48	10.28 ± 0.59	13.81 ± 2.77	474.52 ± 42.69

Measured values are expressed as mean ± SD of triplicates.

antibody. Oxidative DNA damage was assayed by measuring the 8-OHdG content at 450 nm.

Statistical analysis

All calculations and standard deviation between the replicates were done using the graphing software, SigmaPlot (version 10.0). Whether differences between the treatments were statistically significant was determined using Student's t-tests at the 95% confidence level.

Results and discussion

Characterization of biochars

Some of the physico-chemical characteristics, i.e. pH, EC, yield, moisture, ash content, mobile, and resident matters of BCs derived from perilla, sesame, and pumpkin seeds are shown in Table 1. The pH and EC values for BC produced at 550 °C ranged from

7.81-10.30 and 0.55-1.06 mS/cm, respectively, which were higher than those at 300 °C. The BCs produced from perilla and sesame meals at 550 °C showed high pH values over 10.0, and it reduced to 7.96 and 8.18, respectively, when amended with artificial soil at an application rate of 5%. Also, the EC values of BCs produced at 550 °C were reduced to 0.22 and 0.43 mS/cm, respectively, when amended with soil. The proportion of yield and mobile matter in the selected BCs at 550 °C were 1.2-2.5 times lower than those at 300 °C. In contrast, the ash and resident matter in BCs at 550 °C were increased to a maximum of two folds compared to those at 300 °C. The moisture content at both temperatures of BCs derived from perilla, and sesame meals, and pumpkin seed were similar.

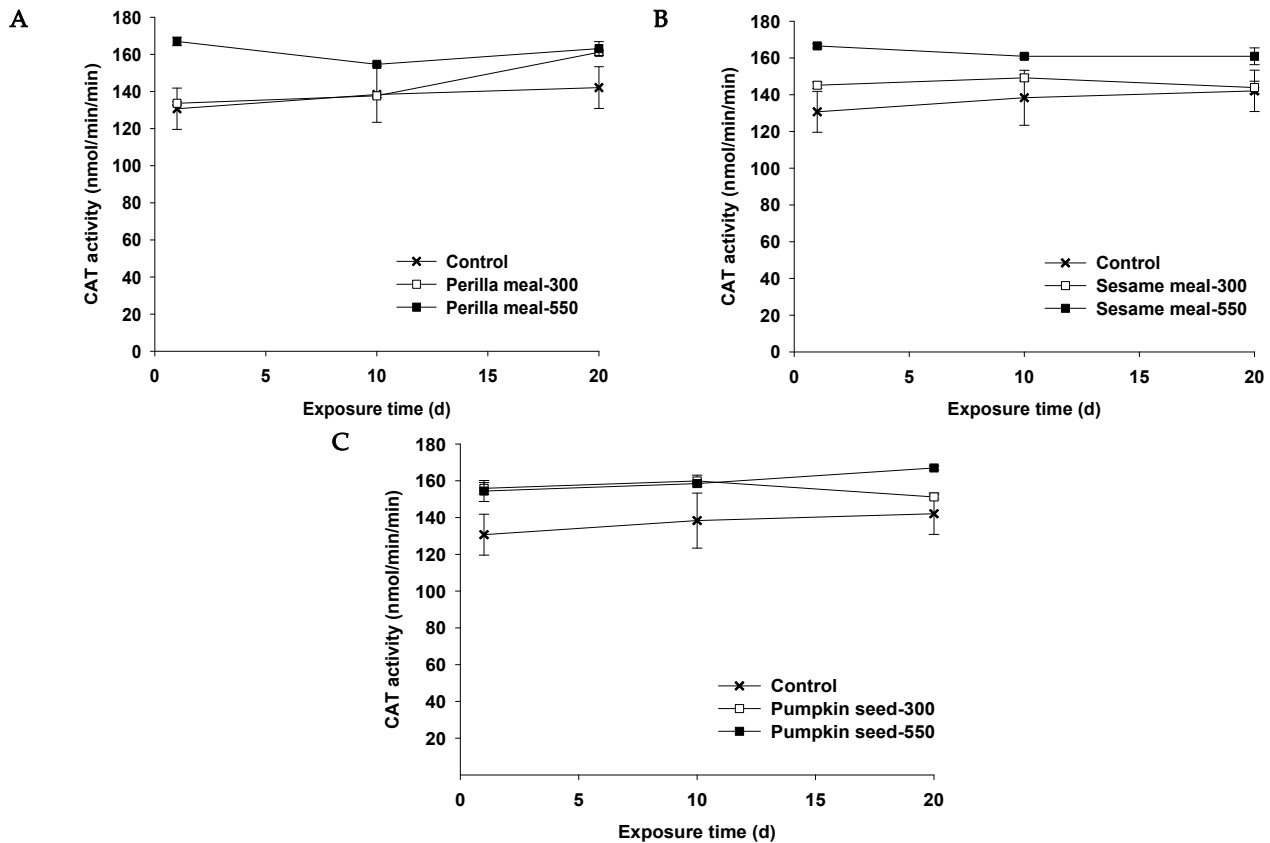


Fig. 1. Catalase measurements in earthworm tissues in control and soil amended with biochar produced from perilla meal (A), and sesame meal (B), and pumpkin seed (C) at 300 and 550°C during the 28 days incubation period. The catalase activity was estimated by measuring the amount of formaldehyde determined at 595 nm, and each value was expressed as mean \pm standard deviation of triplicates.

Trace metal(loid) concentrations

The contents of trace metal(loid)s in BCs derived from perilla, and sesame meals, and pumpkin seed are shown in Table 2. The BCs produced at 300 and 550°C showed high contents of zinc (Zn), copper (Cu), chromium (Cr), and cadmium (Cd) in the range of 149.92-474.52 mg/kg, 60.32-131.60 mg/kg, 3.01-~12.5 mg/kg, and 0.05-2.77 mg/kg, respectively. These contents were approximately 2-3 times higher in BCs pyrolyzed at 550°C than those at 300°C. No significant difference in lead (Pb) contents was observed in BCs produced at both temperatures. The arsenic (As) contents were not detected in BCs pyrolyzed at 300 and 550°C. In pumpkin seed BC, the contents of Cd were not detected and the Ni contents were two folds higher at 300°C rather than at 550°C.

Effect of biochar on the growth, mortality, and cocoon production of earthworms

The survival, average weight loss, and cocoon numbers in the earthworms were determined after 28 days of exposure to soil amended with 5% BC

derived from perilla, and sesame meals, and pumpkin seed at 300 and 550°C (Table 3). All the worms exposed to BC-amended soil for 28 days survived. Average weight loss (%) of *E. fetida* was slightly increased by the exposure of soil amended with BC₅₅₀. Approximately, 2-fold average weight losses were observed in worms exposed to BC₅₅₀ from perilla and sesame meals than BC₃₀₀. The average weight losses of earthworms were in the range of 12.9-22.1% in perilla meal, 9.4-16.2% in sesame meal at 300 and 500°C, respectively, and 17.6% in pumpkin seed at both temperatures. No significant difference in average weight loss was observed among the selected BCs ($p > 0.05$). Cocoon numbers of *E. fetida* showed similar values in the ranges of 3.02 ± 0.58 - 3.63 ± 0.68 for all the treatments. After the 28 days incubation period, there was a notable increase in the pH in soils amended with BCs. Liesch *et al.* (2010) also noticed an increase in pH after the earthworm toxicity test in the presence of pine chip and poultry litter biochars. They also observed mortality and weight loss in worms present in poultry litter BC amended soils,

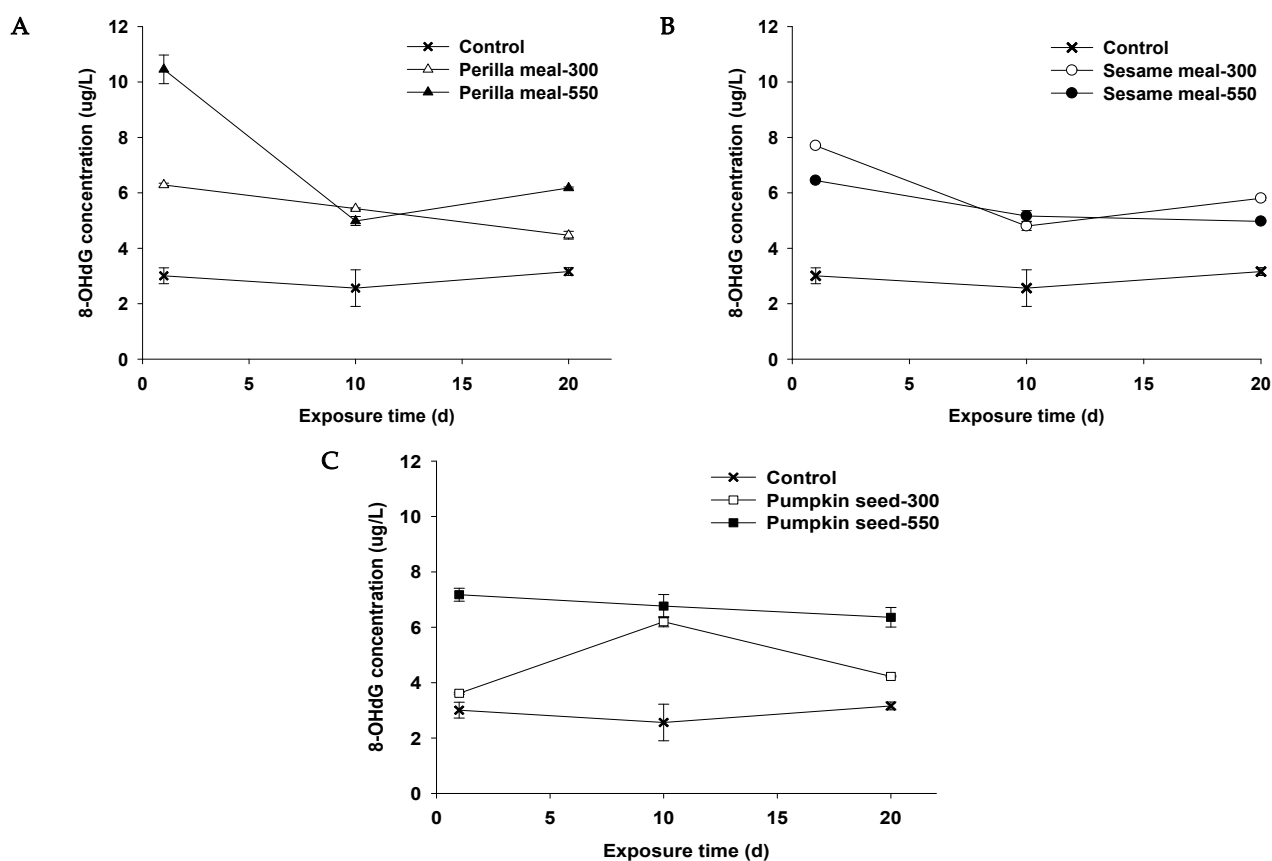


Fig. 2. 8-hydroxydeoxyguanosine measurements in earthworm tissues in control and soils amended with biochar produced from perilla meal (A), and sesame meal (B), and pumpkin seed (C) at 300 and 550°C during 28 days incubation period. Measured value indicates the mean \pm standard deviation of triplicates. Statistically significant difference in 8-hydroxydeoxyguanosine concentrations was not observed between control soil and biochar-amended soil.

however, no significant effects in pine chip biochar treatment were recorded. Li *et al.* (2011) reported that BC mixture at 10 and 20% in artificial soil significantly increased weight loss compared to control, but did not show significant effects on reproduction.

Enzyme activity measurements

The CAT enzyme activity performed to assess the oxidative cellular damage of *E. fetida* due to ROS production were estimated in control and soil amended with BC₅₅₀ during the 28 days exposure (Fig. 1). The worms exposed to BCs prepared from perilla and sesame meals showed slight induction of CAT activities, but time-dependent variations were

Table 3. The survival, average weight loss (%), and cocoon numbers of earthworms and soil pH after 28 days exposure to soil amended with various biochars

Biochars	pH	Survival (%)	Initial weight (g)	Average weight loss (%)	Cocoon number/worm
Control	-	100	0.52 \pm 0.06	6.54 \pm 0.06	3.63 \pm 0.68
Perilla meal-300	7.81 \pm 0.03	100	0.56 \pm 0.02	12.95 \pm 0.03	3.27 \pm 0.78
Perilla meal-550	8.32 \pm 0.06	100	0.57 \pm 0.09	22.12 \pm 0.11	3.20 \pm 0.80
Sesame meal-300	7.78 \pm 0.05	100	0.60 \pm 0.07	9.42 \pm 0.07	3.13 \pm 1.07
Sesame meal-550	8.51 \pm 0.08	100	0.58 \pm 0.07	16.21 \pm 0.08	3.28 \pm 0.12
Pumpkin seed-300	7.32 \pm 0.03	100	0.57 \pm 0.05	17.63 \pm 0.04	3.02 \pm 0.58
Pumpkin seed-550	7.86 \pm 0.04	100	0.58 \pm 0.07	17.62 \pm 0.06	3.34 \pm 0.78

not observed. Similar CAT activities on earthworm tissues during the 28 days exposure were observed between control and soil amended with BCs produced at 300°C. The CAT activities were enhanced from 160 to 170 nmol/min/mL on *E. fetida* exposed to BCs produced at 550°C. The earthworm tissues in BC₅₅₀ and BC₃₀₀ derived from pumpkin seed showed no significant differences ($p>0.05$) in CAT activities, and only showed a slightly higher induction, to a maximum of 160 nmol/min/mL than the control soil. Li *et al.* (2011) reported that superoxide dismutase (SOD) activity in earthworm tissue did not show significant differences in soil amended with 20% BC during the 14 days test Li *et al.* (2009) noticed increased catalase and cellulose activities in Cd- and Pb-treated *E.fetida* which indicated toxicant induced DNA damage.

The 8-OHdG activity as a biomarker of oxidative DNA damage on the earthworm *E. fetida* in control and soil amended with BCs were measured during the 28 days exposure (Fig. 2). The results indicated increased levels of 8-OHdG concentrations in worms in BC₃₀₀ and BC₅₅₀ soils than the control soil during the 28 days exposure. This indicated that enhanced 8-OHdG concentration of *E. fetida* tissue exposed to BCs was associated with stimulated oxidative DNA damage. No significant difference in 8-OHdG concentrations between BC₃₀₀ and BC₅₅₀ exposed worms was observed. However, the worms in soil amended with BC₅₅₀ produced from perilla and sesame meals, and pumpkin seed increased the induction of 8-OHdG with a range of 5-10 µg/L, which slightly induced to a maximum of 2-folds compared to soil amended with BC₃₀₀ ($p>0.05$). This may be due to the high contents of trace metal(loid)s in BC₅₅₀ over BC₃₀₀ (Table 2). Greater concentrations of 8-OHdG were observed by Li *et al.* (2011) in soil amended with BC produced from woodchips at 550°C than 300°C. The BCs pyrolyzed over 500°C contain high concentrations of trace metal(loid)s, which are known to cause oxidative stress in earthworms (Weyers and Spokas, 2011).

Conclusions

This study examined the survival, growth, reproductive tests, and oxidative DNA damage tests on earthworm *E. fetida* in artificial soil amended with 5% BCs pyrolyzed at 300 and 550°C from perilla and sesame

meals, and pumpkin seed. Results indicated that weight loss in earthworms slightly increased in BC-amended soil than the control after 28 days exposure. The 8-OHdG level and CAT activity increased in earthworms exposed to BCs-amended soil compared to control. Moreover, the 8-OHdG concentrations in BCs amended soil at 500°C were greater than those at 300°C. These observations were attributed to the high amounts of trace metal(loid)s and also an increased pH in BCs. This study suggests that it is necessary to investigate the long-term impact of BCs on earthworms in agricultural fields. As BC characteristics depend on the feedstock and conditions of pyrolysis, toxicity screening of BCs, prior to land application is recommended.

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