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Biochemical and Agronomic Responses of Soybean (*Glycine max* L. Merrill) to Spent and Deoiled Bleaching Earth of NPK Fertilization on Filler Basis

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ABSTRACT

Spent bleaching earth (SBE) is the largest waste produced by the palm oil industry. However, according to several studies, SBE and its recovery product DBE have the potential as filler materials in NPK fertilizers. This study examines the influence of NPK fertilizer with SBE and DBE as filler materials on soybean plants' biochemical and agronomic properties. The field-based experiment was done in a single-factor randomized complete block design with 4 replicates. We tested fertilizers of 10% bentonite clay mineral using NPK on a filler basis (control), 5% bentonite clay mineral with 5% SBE of NPK on a filler basis, and 5% bentonite clay mineral with 5% DBE using NPK on a filler basis. The variables observed include soil chemical properties after applying fertilizer, which involves the concentrations of several heavy metals. Biochemical characteristics, including the content of hydrogen peroxide (H_2O_2) and peroxidase (POD), superoxide dismutase (SOD) activity, malondialdehyde (MDA), relative electrolyte leakage (REL), total phenolic content, and proline content. The agronomic characteristics of soybean plants, including root and shoot dry weight. The data were analyzed using ANOVA and tested using the least significant difference test at a 95% confidence interval. The results indicated that materials of SBE and DBE could partially substitute the filler elements in bentonite clay mineral of NPK fertilizer on a filler basis, and they had the same

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influence in SOD activity, H_2O_2 content, POD, MDA, REL, total phenolic, proline and root dry weight and shoot of soybean plants.

Keywords: Agronomic, biochemical, NPK, soybean, spent bleaching earth

INTRODUCTION

Spent bleaching earth (SBE) is the largest waste produced by the palm oil industry. The increase in palm oil production has resulted in increased waste from bleaching, contributing to the production of SBE. According to the Government Regulation of the Republic of Indonesia No. 101/2014 concerning the Management of Hazardous and Toxic Wastage, SBE is classified as category 2 hazardous and toxic (B3) waste (Pasaribu & Sukandar, 2017).

SBE, along with the recovery, could produce deoiled SBE (DBE), and it contains some heavy metals, such as Cu, Zn, Cd, Ag, and Ni, when re-purified by removing the oil content (Loh et al., 2015). These elements cause the materials of SBE and DBE to be combustible and cause environmental pollution because of the content of these heavy metals. It highlights the need to manage SBE and DBE waste and how to reuse them. Reusing them could help address B3 waste issues because the waste could be transformed into economically useful materials, including the replacement of filler elements in NPK fertilizers. Bentonite (brown) clay is frequently used as filler and has identical properties to bleaching earth (BE) (Anugrah et al., 2020; Wisnubroto et al., 2021).

However, the effects of using SBE and DBE as a partial substitution of NPK fertilizer filler on the environment and plants still require further evaluation, considering that both materials contain some essential and non-essential heavy metals (Purba et al., 2020; Wisnubroto et al., 2020). Plants can respond positively or negatively to changes in the environment, depending on the type and cultivar. This response can be seen from changes in biochemical and agronomic processes in plants (Wisnubroto et al., 2023). Pratap et al. (2012) and Zakiah et al. (2017) state that soybeans occupy a premier position among agricultural crops, being the most important source of good-quality concentrated proteins as well as vegetable oil. The soybean plant is known to be slightly sensitive to environmental conditions, especially toxic elements such as heavy metals (Taufiq & Sundari, 2012). This study used soybean plant as a model plant to determine whether SBE and DBE materials can be used to replace some of the filler components in NPK fertilizer based on biochemical and agronomic processes in plants.

MATERIALS AND METHODS

Study Area

The study was carried out between October 2018 and January 2019 at the Agro-Technology Innovation Center (PIAT), Universitas Gadjah Mada, Yogyakarta, Indonesia (Figure 1). The

study was located 124 m above sea level in terms of altitude. According to the classification by Oldeman, Berbah is classified into a C3 climate, consisting of 5 to 6 rainy months and 5 to 6 dry months, respectively (Harmoni, 2014). The materials used in the experiment were cultivar. Grobogan soybean plants were cultivated extensively around the area. The other materials were NPK fertilizer (15:15:15) with 10% bentonite clay mineral, 5% bentonite clay mineral with 5% DBE, and 5% mineral clay with 5% SBE as filler elements.

The experiment was done in one-factor field within randomized complete block design (RCBD) with 4 replicates. 10% of bentonite clay using NPK fertilization on filler basis (control), 5% of bentonite clay with 5% of SBE using NPK fertilization on filler basis, and 5% of bentonite clay with 5% of DBE using NPK fertilization on filler basis were the treatments tested. The SBE and DBE were obtained from PT. Sentana Adidaya Pratama (SADP), a part of the Wilmar Group Indonesia. For all treatments, NPK fertilizers were administered two times: on the 14th and 35th day after planting with the amount of 150 kg/ha and 225 kg/ha, consecutively. A deep placement scheme with ±5 cm in distance from the plant roots was used, and then the fertilizer was spread to avoid disturbing the roots.

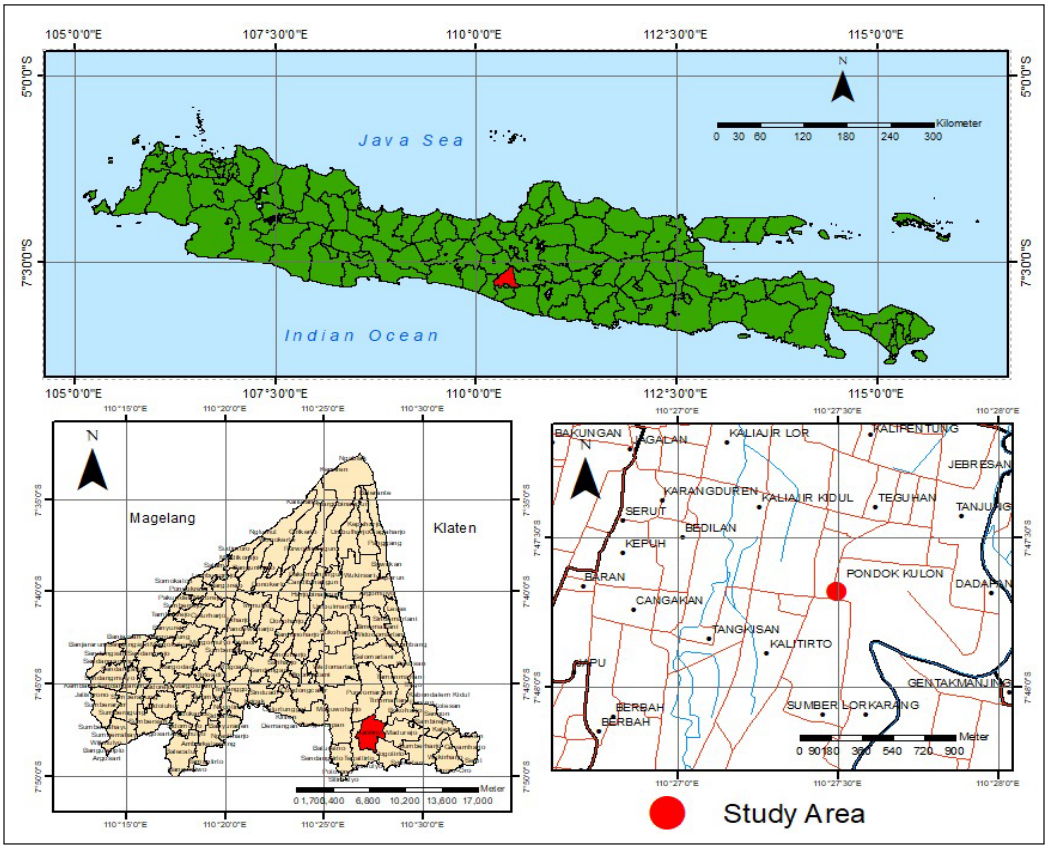


Figure 1. Research location at the Agro-Technology Innovation Center (PIAT) at Universitas Gadjah Mada, Yogyakarta, Indonesia

Procedures

The variables observed were soil chemical properties after being fertilized. These included the concentrations of Cu, Zn, Cd, Ag, and Ni. The observation was conducted 21 days after planting. Biochemical characteristics, including the content of hydrogen peroxide (H_2O_2) and peroxidase (POD), activity of superoxide dismutase (SOD), malondialdehyde (MDA), relative electrolyte leakage (REL), total phenolic content and proline content, were observed 70 days post planting. The agronomic characteristics of soybean plants, including root and shoot dry weight, were observed 70 days post-planting.

Soil Chemical Properties. Soil chemical properties observed in this study were the concentrations of Cu, Zn, Cd, Ag, and Ni using diethylene triamine penta acetic acid (DTPA) extract described by Eviati and Sulaeman (2009). DTPA extract can dissolve metal ions in the form of chelate compounds. DTPA solution has the strongest chelating power to extract iron and other metals at a pH of 7.3. A total of 10 g of a good sample of soil (<2 mm) was weighed and added with 20 mL of DTPA extracting solution, then shaken with a shaking machine for 2 hours. The suspension was filtered or centrifuged to obtain a clear extract. Each element was then measured with the AAS tool (Equation 1).

The concentrations of heavy metals (ppm)
= ppm of the curve × mL of the extract 1000 mL^{-1} × 1000 g (g sample)$^{-1}$ × fp × fk
= ppm of the curve × 20 1000^{-1} × 1000 10^{-1} × fp × fk
= ppm of the curve 2 × fp × fk [1]

Remarks:
ppm = the sample concentration received out of the relationship curve of the standard range and the reading after blank correction
fp = dilution factor (if any)
fk = correction factor of moisture content = 100 / (100 - % moisture content)

The Concentrations of Heavy Metals in Plant Tissues. Concentrations of Cu, Zn, Cd, Ag, and Ni in the tissue were measured on leaves 70 days after planting. These heavy metals can be extracted by wet ashing by mixing the concentration of HNO_3 and $HClO_4$ acids. Heavy metal concentrations in the extract were measured with AAS (Eviati & Sulaeman, 2009).

The concentration of heavy metals was measured by carefully weighing 2.5 g of good samples of plant <0.5 mm. The samples were placed in a digest tube, added with 5 mL of nitric acid concentration, and left overnight. The following day, the samples were heated at 100^{o}C for 1 hour and 30 minutes and cooled. Then, 5 mL of nitric acid concentration and 1 mL of perchloric acid concentration were added. The samples were then heated to 130^{o}C for 1 hour and 150^{o}C within 2 and a half hours until yellow steam disappeared. The heating time was prolonged when yellow steam continued to form after 2.5 hours. Once

the yellow steam disappeared, the temperature rose to 170°C for 1 hour and then to 200°C for 1 hour until white vapor was formed. Destruction was finished when a white precipitate or the remainder of a clear solution of about 1 mL was formed. The extract was cooled and diluted with 25 mL of ion-free water. Then, it was shaken until homogeneous and left overnight. The clear extract measured the concentration (ppm) of Cu, Zn, Cd, Ag, and Ni using the SSA method on the flame method (Equation 2).

The level of heavy metals (ppm)
= ppm of the curve × mL of the extract 1000 mL^{-1} × 1000 g (g sample)$^{-1}$ × fp x fk
= ppm of the curve × 25 mL 1000^{-1} mL × 1000 g 2.5^{-1} g sample × fp × fk
= ppm of the curve × 10 × fp × fk [2]

Remarks:
ppm = the sample concentration received out of the relationship curve of the standard range and the reading after blank correction
fp = dilution factor (if any)
fk = correction factor of moisture content = 100 / (100 - % moisture content)

Biochemical Characteristics. SOD activity testing was carried out based on the autoxidation of pyrogallol developed by Marklund and Marklund (1974). Briefly, 50 mM of Tris-HCl buffer with a pH of 8.2 and 1 mM 25 EDTA were used as a medium of reaction. They were combined with a 40-60 mg sample of protein extract and mixed with 100 µL 0.2 mM of pyrogallol solvated in 50 mM of PPB pH 6.5 to initiate the reaction. A decrease in the absorbance of pyrogallol was monitored at 420 nm. The activity of SOD was conveyed in units per mg of protein (U mg^{-1} protein).

The content of H_2O_2 was measured according to the spectrophotometric method developed by Alexieva et al. (2001). Fresh leaves of 0.5 g were crushed and put into a test tube, and then 5 mL of 0.1% (w / v) trichloroacetic acid (TCA) was added for homogenization. A total of 0.5 mL of supernatant was placed in the test tube, and then 0.5 mL of 100 mM potassium phosphate buffer and 2 mL of potassium iodide (KI) reagent (1 M KI w / in H_2O) were added. The solution was then left in a dark place for an hour. Next, the sample was put into the cuvette, and the absorbance was read using Spectronic 21D at a wavelength of 390 nm. One-tenth of one percent of TCA was utilized as blank. The content of H_2O_2 was calculated with the H_2O_2 standard curve equation whose concentrations were known. The standard curve was determined using different concentrations of pure H_2O_2. A 1000 ppm of H_2O_2 stock solution was prepared and diluted to a certain concentration, and the absorbance was then read using Spectronic 21D at a wavelength of 390 nm. The H_2O_2 content is expressed in parts per million (ppm).

POD content was determined according to the spectrophotometric method developed by Zhang et al. (1995). The fresh leaves were crushed, then 1 gram of the crushed leaves

was taken and added with 1 mL phosphate buffer with a pH of 7, then placed into a 2 mL tube. The solution was centrifuged at 1500 rpm for 10 minutes at 4°C. Then reagents, consisting of 1000 µL dH_2O, 160 µL potassium phosphate buffer with a pH of 6 at 20°C, 80 µL peroxide solution, 180 µL pyrogallol solution, and 100 µL supernatant sample, were prepared. The solution mixture was then inserted into the cuvette and read using a spectrophotometer with an absorbance of 420 nm. It was then observed three times with intervals of 30 seconds. The POD content is expressed in units per mL of the enzyme (U mL^{-1} enzyme) and can be calculated using the following Equation 3:

$$\text{POD content} = \frac{(\Delta A420/20 \text{ sec test sample} - \Delta A420/20 \text{ sec blank}) \times 1.55 \times 1}{(12)\,(0.1)} \quad [3]$$

Lipid peroxidation was determined with MDA as the final product using the thiobarbituric acid (TBA) method developed by Cakmak and Horst (1991). One g of fresh leaves was crushed and homogenized with 2 mL of 0.1% (w / v) trichloroacetic acid (TCA) solution. Next, 1.5 ml of the solution was centrifuged at 15,000 g for 10 minutes. A half mL supernatant was added to 1.5 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was shaken and incubated in a water bath at 90°C for 20 minutes. The tube for testing was placed in a beaker filled with ice, which was centrifuged at 10,000 g within five minutes after cooling down. The supernatant was loaded into the cuvette, and the absorbance was read with Spectronic 21D at 532 and 600 nm wavelengths. An absorption coefficient of 155 $mM^{-1}cm^{-1}$ calculated MDA. The blank used was 0.1% TCA. The MDA content is expressed in µM per leaf fresh weight (µM $fresh^{-1}$ weight of leaves) that can be calculated using the following Equation 4:

$$\text{MDA} = \frac{((\text{difference in absorbance of 532 and 600 nm})/155 \text{ mM}^{-1}\,\text{cm}^{-1} \times 10^6))}{\text{Fresh weight of leaves}} \quad [4]$$

The leakage of electrolytes can be measured by measuring the concentrations of electrolytes leaking from the cell using REL. REL levels were determined according to Dionisio-Sese and Tobita's method (1998). One-tenth of a g of fresh leaves were sliced with a length of 5.0 mm and put in a testing tube with 10 mL of deionized distilled water. Next, the tubes were enclosed in plastic and put in a water bath at a temperature of 32°C. After 2 hours, the first electrical conductivity of the medium (EC_1) was measured using an electrical conductivity meter (CM-115, Kyoto Electronics, Kyoto, Japan). The sample was then autoclaved at 121°C for 20 minutes to destroy the tissue and release all electrolytes. The sample was cooled to 25°C, and the last electrical conductivity (EC_2)

was measured. REL levels are expressed in percent (%) that can be determined by the following Equation 5:

$$REL = \frac{EC_1}{EC_2} \times 100\% \quad [5]$$

Total phenolic content was measured using a visible spectrometric method following the procedure of Chun et al. (2003). The plant leaves were put in an oven for 48 hours at 40°C, then mashed (0.05 g) and put into a test tube. Four-tenths of a mL of Folin-ciocalteu was added, left for 5–8 minutes, added with 4 mL of 7% Na_2CO_3, and filtered with filter paper. The mixing result was placed in a 10-ml volumetric flask, and aqua bidestilata was added to the limit of 10 ml and left to stand for 2 hours. The mixture was then put into the cuvette, and the absorbance was read at a wavelength of 765 nm using Spectronic 21D. The blank used was aquabidest. The total phenolic content was calculated using the standard curve equation for total phenolics whose concentrations were recognized. The total phenolic standard curve was determined using different concentrations of pure phenol. A 1000 ppm phenol stock solution was prepared and diluted to definite concentrations, and the absorbance was read with Spectronic 21D at 765 nm of wavelength. The total phenolic is expressed in parts per million (ppm).

Proline content was determined by a method developed by Bates et al. (1973). The fresh leaves (0.5 g) were pounded with a mortar in 10 ml of 3% sulfosalicylic acid solution. The collision leads to the results being by Whatman filter paper. Next, a ninhydrin acid solution was created by dissolving 1 g of ninhydrin in 24 ml of glacial acetic acid, and the test tube was kept warm until the solution turned blue. A total of 2.5 ml of phosphoric acid plus 5.5 ml of distilled water was added to the ninhydrin solution and was heated until dissolved. Two ml of the filtrate was reacted with 2 ml of ninhydrin acid and 2 ml of glacial acetic acid in a test tube at 100°C for 1 hour. The reaction was finalized by placing the test tube into a beaker filled with ice. The mixture was extracted with 4 ml of toluene and shaken with a stirrer for 15–20 seconds. The red tolerant comprising proline at the top was sucked with a pipette. The absorbance of the solution was read with a Spectronic 21D at a wavelength of 520 nm. Proline content is expressed in µmol proline per gram (µmol proline g^{-1}) that can be determined by the following Equation 6:

$$\text{Proline content} = 64.3649 \times \text{absorbance reading} + (-5.2987) \times 0.347 \quad [6]$$

Agronomic Characteristics. The agronomic characteristics are shown in the form of plant biomass, including root and shoot dry weight. Plant dry weight showed how organic compounds that were integrated from inorganic substances by plants were accumulated

and acquired after the drying plant segments at 80°C in the oven for ±48 hours until they reached sustained weight. It could be determined by measuring the weight of the plants a few times at 24-hour intervals. Weight measurement uses analytical scales, and the weight is expressed in grams per plant (g plant^{-1}).

Data Analysis

The data gathered were tested with variance (ANOVA) analysis. Tests of data assumptions on the normal distribution and homogeneity were previously carried out. Based on the results of the ANOVA, the data showed significant differences between treatments and were tested using the least significant difference (LSD) test at a 95% confidence interval. Data analysis was done using SAS version 9.4 software.

RESULTS AND DISCUSSIONS

Soil Chemical Properties

Naturally, soil accommodates diverse heavy metals (Alloway, 1995). Handayanto et al. (2017) stated that they are originally from parent material weathering done at low levels and are generally not dangerous. In addition, heavy metals in the soil can also originate from human activities, usually called anthropogenic activities, producing greater concentrations of metals than natural sources. Metal pollutants from such sources include inorganic or organic fertilizers, mining, and pesticides (Erfandy & Juarsah, 2014).

NPK fertilizers used in this study: Cu, Zn, Cd, Ag, and Ni are heavy metals known to contaminate the soil indirectly when used. Some of these are among the priority metals, such as Cu, Ni, and Zn. The 5% SBE and DBE used as fillers in NPK fertilizer are thought to have higher heavy metallic content compared to the 10% clay mineral added as filler in NPK fertilizer because SBE and DBE are derived from bleaching earth materials, which are not only used as a bleaching agent but are also used to reduce other undesired elements such as heavy metals. The concentrations of heavy metals in the soil at the research site after being fertilized are shown in Table 1.

The analysis showed that the three treatments did not significantly influence the concentrations of heavy metals tested. Furthermore, Alloway (1995) reported that all kinds of heavy metals concentrations in the research site soil were still lower than the critical limit, except for Ag (Table 1). High concentrations of Ag may damage plants as they inhibit fertilization by inhibiting cell elongation in roots. They also damage vacuoles and cell walls and reduce magnesium, phosphorus, and sulfur nutrient absorption, eventually disrupting the formation of roots (Shofi, 2017).

The toxic limit of Ag varies in diverse species of plants, ranging from extremely hazardous to slightly hazardous. In soybean plants, applying Ag nanoparticles by 30 ppm

Table 1
Concentrations of heavy metals in the soil post-NPK fertilizer treatments with diverse filler materials 21 days after planting

Treatment	Ag	Cd	Cu	Ni	Zn
	---------- ppm ----------				
NPK + 10% of bentonite clay mineral	3.88 a	undetected	55.35 a	undetected	42.58 a
NPK + 5% of bentonite clay mineral + 5% of SBE	4.97 a	undetected	57.81 a	undetected	44.37 a
NPK + 5% of bentonite clay mineral + 5% of DBE	4.42 a	undetected	53.13 a	undetected	42.14 a
CV (%)	17.08	-	2.36	-	2.29
Critical limit of heavy metals in the soil (ppm)*	2	75–100	60–125	100	70–400

Note. The same letters that follow the means do not differ significantly in accordance with a test of least significant difference (LSD) at a 95% confidence interval; the concentrations of Cd and Ni after treatments were undetected or lower than the detection limit (detection limit of Cd = 0.01 ppm and Ni = 0.25 ppm); *Critical limit of heavy metals in the soil by Alloway (1995)

of concentration to the soil does not present any significant influence on the root fresh weight, even though it has a tendency to decline with the increase of dosages given (Li et al., 2017). The presence of Ag in the soil at the research site, pre- and post-treatments, was still safe for the growth and development of the soybean plants.

Concentrations of Heavy Metals in Plant Tissues

In plants, heavy metals could enter the tissue via roots and stomata (Alloway, 1995). Heavy metals, such as Cu, Ni, and Zn, are vital components that plants need in small proportions. However, when they are highly concentrated, they can disturb plant growth (Deswati et al., 2020; Rusnam et al., 2013). In contrast, Ag, Cd, Cr, and Pb are not significant elements of the soil as they disturb plant growth (Harmiwati et al., 2015; Janoušková et al., 2006; Rusnam et al., 2022). The concentrations of heavy metals in the tissues of soybean plants are shown in Table 2.

It has been shown that giving such treatments had no significant influence on the concentrations of heavy metals in plant tissues 70 days post-planting, except Cu and Zn (Table 2). The concentrations of all metals in the plant tissue were still lower than the soybean plants' critical limit. The presence of Cd was undetected, indicating that the concentration was lower than 0.01 ppm. This result is at a constant ratio to the concentration of Cd in the soil in this study (Table 1).

The concentration of Zn in DBE used as filler material in NPK treatment had a significant difference and was higher than in the control treatment. Sharma et al. (1994) stated that Zn plays a role as a cofactor for enzymes which functions as the antioxidant, superoxide dismutase (SOD). Zn, along with Cu, binds to the SOD enzyme to create

Table 2
Concentrations of heavy metals in soybean plant tissues as a result of diverse fertilization treatments 70 days after planting

Heavy metal element	Treatment	Concentration of heavy metal elements in plant tissues (ppm)	CV (%)	Critical limit of heavy metal element content in soybean (ppm)
Ag	BC	0.01 a	20.40	1–30 Li et al. (2017)
	SBE	0.32 a		
	DBE	0.46 a		
Cu	BC	8.88 b	7.93	100–500 Nair and Chung (2014)
	SBE	11.02 ab		
	DBE	14.13 a		
Ni	BC	0.94 a	31.51	50–100 Fitriani et al. (2019)
	SBE	0.25 a		
	DBE	2.28 a		
Zn	BC	14.17 a	9.02	150–200 Fageria et al. (1997)
	SBE	17.85 ab		
	DBE	26.70 a		

Note. The same letters that follow the means do not differ significantly in accordance with a test of least significant difference (LSD) at a 95% confidence interval. The concentration of Cd was undetected or lower than the detection limit (detection limit of Cd=0.01 ppm). BC = NPK + 10% of bentonite clay, SBE = NPK + 5% of bentonite clay with 5% of SBE, DBE = NPK + 5% of bentonite clay with 5% of DBE

CuZnSOD, which frequently exists in cells of plants to increase plant oxidative stress tolerance. It is possible that the concentrations of Zn increase in plant tissue treated with DBE as filler material in NPK fertilization since the nano-sized pores of materials resulting from the deoiling process are filled with minerals, allowing the plants to supply these components.

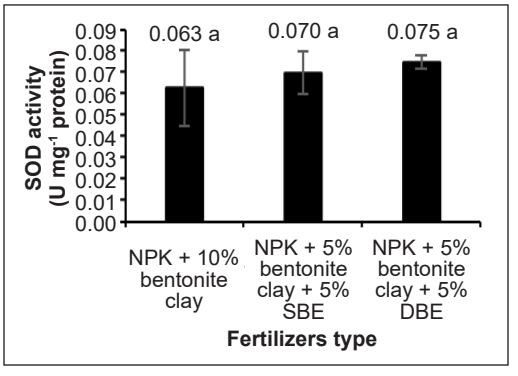
Biochemical Characteristics

At the molecular level, high concentrations of heavy metals in plant cells can disrupt the balance of cellular redox reactions and influence oxidative stress directly or indirectly, depending on their chemical characteristics (Fargasova, 2001; Smeets et al., 2009). Metals classified as active redox, such as Cr, Cu, Mn, and Fe, can induce the production of reactive oxygen species (ROS) directly through the reaction of Fenton and Haber-Weiss (Yruela, 2005). On the contrary, inactive redox metals, for example, Cd, Ni, Hg, Zn, and Al, only induce the production of ROS via indirect methods, such as inhibiting antioxidant enzymes or stimulating ROS-producing enzymes (NADPH oxidase) (Bücker-Neto et al., 2017; Smeets et al., 2008; Stoyanova & Doncheva, 2002). During the process, reactive oxygen species (ROS) are created and thus change the balance of redox into the pro-oxidative side.

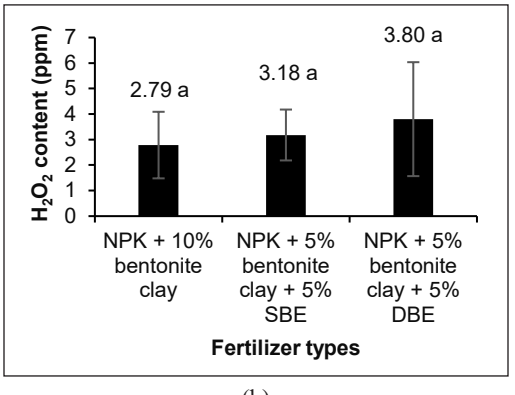
In general, plant cells continuously form free radical ions in the form of ROS as a by-product of aerobic metabolism that takes place in various cellular compartments such as cell walls, cytoplasm, peroxisomes, mitochondria and chloroplasts (Vianello et al., 2007). The formation of free radical ions such as ROS will increase when plants experience both abiotic and biotic stress. The accumulation of free radical ions in the form of ROS at high concentrations will damage cellular and macromolecular components, including the plasma membrane, nucleic acids, and proteins. The formation of ROS also has a function as an effector and regulator in the process of programmed cell death (Malecka et al., 2014).

In dealing with this oxidative stress, plants have enzymatic defense systems like superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). In addition, plants also have a non-enzymatic defense system that is antioxidant, including ascorbic acid (AsA), tocopherols, phenolic, alkaloids, proline, and carotenoid compounds (lignin, tannins, flavonoids), which act as ROS fasteners (Sharma et al., 2012). According to Kumalaningsih (2007), antioxidants are substances with molecular structures that possess the ability to supply free electrons to free radical molecules without being disrupted in their entirety and to break the free-radical chain reaction. The two defense systems work synergistically to neutralize the toxic effects of ROS compounds so that ROS is only present in small amounts needed to maintain normal cell function.

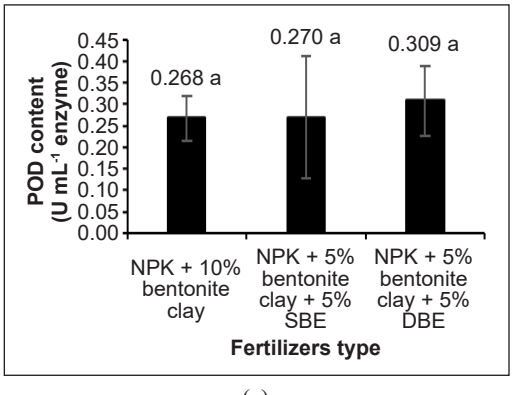
SOD activity, H_2O_2 content, and POD content on the leaves were observed 70 days after planting (Figure 2).



(a)



(b)



(c)

Figure 2. (a) SOD activity, (b) H_2O_2 content, and (c) POD content as influenced by NPK fertilization with various filler materials 70 days after planting. Remark: The data presented are standard deviation ± mean; the same letters that follow the means do not differ significantly in accordance with a test of least significant difference (LSD) at a 95% confidence interval

The results found that those treatments did not significantly influence the SOD activity, H_2O_2 content, and POD content. The findings suggest that the use of SBE and DBE as filler materials did not influence the three variables observed. However, the use of 5% SBE and DBE tended to increase SOD activity, H_2O_2 content, and POD content by 11.11% and 19.05%, 13.96%, and 36.35%, and 0.75% and 15.30%, respectively, compared to control treatment (Figure 2).

This increase was most likely due to the presence of Zn metal in the 5% SBE and DBE of NPK fertilizer on a filler basis, which was higher than in the 10% clay mineral using NPK on a filler basis (control). It could increase the metal content in the soil and plant tissue. The increase in SOD enzyme activity in the 5% NPK fertilization on the filler basis of SBE and DBE shows that soybean plants are tolerant to the presence of Zn because they respond to the excess of these elements in plants. This increase is directly proportional to the H_2O_2 and the POD enzymes formed.

Superoxide dismutase (SOD) is an enzyme containing the essential metals of Cu and Zn to catalyze several chemical reactions in cells. Oxygen-free radicals in the form of superoxide anions ($O_2^{\bullet -}$), which are formed due to heavy metal stress, will be catalyzed by SOD to form hydrogen peroxide (H_2O_2) and oxygen (O_2) (Löffler & Petrides, 1988).

$$2\,O_2^- + 2\,H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

H_2O_2 is formed in the plant body as a short-term product of a biochemical process and is toxic to cells. As the next defense system, plants will produce antioxidants in the form of POD in response to the accumulation of ROS in the form of H_2O_2 (Békésiová et al., 2008). According to Vicuna et al. (2011), POD is one of the key enzymes that plays a role in maintaining cells against oxidative stress by catalyzing the change of H_2O_2 to water (H_2O). Wang et al. (2008) state that plants that are tolerant of heavy metals will show an increase in POD content when exposed to high concentrations of heavy metals. If this were not the case, H_2O_2 might undergo a Fenton and Haber-Weiss reaction to produce more damaging hydroxyl radicals ($OH^{\bullet}$) (Stadtman, 1992). The reaction is as follows (Cuypers et al., 2013):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- \text{ (Fenton)}$$

$$O_2^{\bullet -} + H_2O_2 \xrightarrow{\text{Fe(III)/Cu(II)}} O_2 + OH^- + OH^{\bullet} \text{ (Haber-Weiss)}$$

However, when exposure levels to heavy metals are too high, these defense mechanisms often fail to neutralize the effects of excess ROS, resulting in increased lipid peroxidation and electrolyte leakage (Howlett & Avery, 1997; Wang et al., 2008; Zhang et al., 2007) that can be used as a marker of the level of cell damage. The products can detect lipid peroxidation, including MDA (malondialdehyde) (Marciniak et al., 2009). MDA is formed

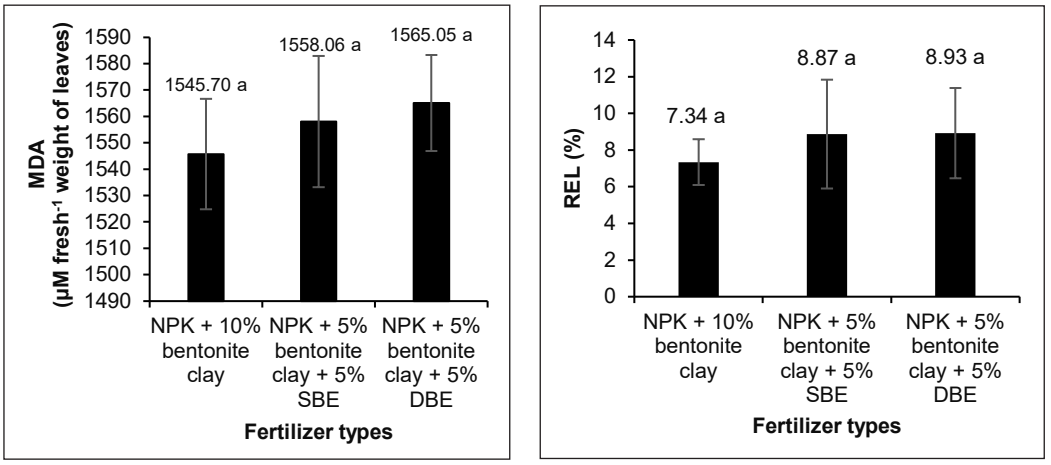
as a result of the reaction between free radicals (ROS) and unsaturated fatty acids (PUFA = Poly Unsaturated Fatty Acid), which is the main element of the cell membrane. The electrolyte leakage can be detected by measuring the amount of electrolyte leaking from the cell using REL (relative electrolyte leakage) as an indicator (Ehlert & Hincha, 2008; Kocheva et al., 2005).

The observations of lipid peroxidation and electrolyte leakage with MDA and REL as indicators were performed on the leaves for 70 days after planting, and the data are presented in Figure 3.

The results indicated that the MDA and REL content had no significant influence on the three treatments. It indicates that the application of SBE and DBE as filler materials in NPK fertilizer does not influence the two variables that are observed. Nevertheless, the use of 5% SBE and DBE in NPK fertilizer had a tendency to increase the MDA and REL content in soybean plants by 0.80%, 1.25%, 20.84%, and 21.66%, respectively, compared to the control treatment (Figure 3).

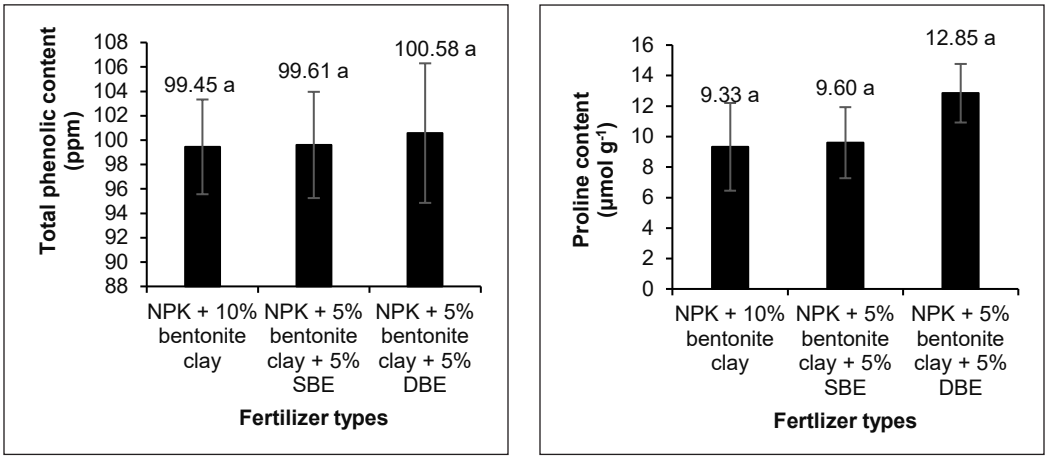
An increase in MDA and REL content influenced by the 5% NPK fertilization on the filler basis of SBE and DBE indicated that soybean plants experienced oxidative stress. This result is at a constant ratio to the SOD activity and the content of H_2O_2 and POD, which also increased (Figure 1). The findings also suggest a decrease does not always follow high antioxidant status in MDA and REL levels. These results indicate that the antioxidants produced are not sufficient in neutralizing the oxidative stress caused by ROS.

In addition to mechanisms using enzymes, plants have non-enzymatic defense systems in managing oxidative stress, including phenolic and proline compounds. The data of phenolic and proline content are presented in Figure 4.



(a) (b)

Figure 3. (a) MDA and (b) REL as influenced by NPK fertilization with various filler materials 70 days after planting. Remark: The data presented are standard deviation ± mean; the same letters that follow the means do not differ significantly in accordance with a test of least significant difference (LSD) at a 95% confidence interval



(a) (b)

Figure 4. (a) Content of total phenolic and (b) proline as influenced by NPK fertilization with various filler materials 70 days after planting. Remark: The data presented are standard deviation ± mean; the same letters that follow the means do not differ significantly in accordance with a test of least significant difference (LSD) at a 95% confidence interval

The results indicated that the total phenolic and proline content had no significant difference in the three treatments. It indicates that the application of SBE and DBE in NPK fertilizers does not influence the two observed variables. Nevertheless, the application of 5% SBE and DBE in NPK fertilizers tended to increase the content of total phenolic and proline in soybean plants by 0.16%, 1.14%, and proline by 2.89% and 37.73%, respectively, compared to the control treatment (Figure 4).

Phenolic substances play a role as antioxidants in plants. Phenolic compounds have one or more hydroxyl groups fixed to the aromatic ring; in other words, they are compounds that have at least one phenyl group (Dhurhania & Novianto, 2019). Phenolic compounds can be classified into distinguished groups by the number of constitutive carbon atoms connected with the basic phenolic structure: benzoic acids, simple phenols, flavonoids and phenylpropanoids (Michalak, 2006).

According to Hanin and Pratiwi (2017), phenolics are compounds produced by plants when responding to environmental stress, for example, heavy metals. Phenolic compound biosynthesis induction was observed in wheat in its reaction to the toxicity of nickel (Díaz et al., 2001) and maize when responding to aluminum (Winkel-Shirley, 2002). The increase in phenolic levels is probably because of the compounds' protective function on heavy metal stress (Brown et al., 1998).

The antioxidant properties of phenolics have a high tendency to chelate metals. Phenolics own hydroxyl and carboxyl groups that can bind metals, especially copper and iron (Jung et al., 2003). In addition, there are other mechanisms underlying the antioxidant abilities of phenolic compounds. Milić et al. (1998) stated that metal ions could break

down lipid hydroperoxide (LOOH) through hemolytic cleavage of O-O bonds and produce alkoxyl lipid radicals, initiating chain reactions of free radicals. Phenolic antioxidants disturb lipid peroxidation by putting these lipid alkoxyl radicals into traps.

No definite proof for a direct role of proline in cellular detoxification against heavy metal stress is found, and many differing opinions regarding how proline reduces metal toxicity are actually found (Mishra & Dubey, 2006). Paleg et al. (1984) stated that proline maintains a favorable water balance in plant tissue by acting as an osmoprotectant. In addition, proline can also act as a stabilizer of protein (Sharma & Dubey, 2004), metal chelating (Farago & Mullen, 1979), inhibitor of lipid peroxidation (Mehta & Gaur, 1999), and neutralizer of free radicals (Alia et al., 2001). Because of its high zwitterionic and hydrophilic properties, proline can protect biomolecules and enzymes (Siripornadulsil et al., 2002). Bertrand and Guary (2002) state that the accumulation of proline in plants is most likely not a direct influence on the stress of heavy metals but the impact of water deficit stress caused by heavy metals.

Biochemically, the phytotoxicity of heavy metals in plants can occur because of these three main factors. These are oxidative stress due to heavy metal induction, a direct effect of metal ions with sulfhydryl groups on protein membranes causing them to malfunction, and inactivation of important enzymes by cation replacement activation with heavy metal ions (Vangronsveld & Clijsters, 1994). Consequently, they may cause functional disturbances in physiological processes as well as anatomical-morphological changes and damage, which can then influence the agronomic characteristics of plants in biomass, including root and shoot dry weight.

Agronomic Characteristics

Plant dry weight shows organic compounds integrated from inorganic substances by plants. According to Shah et al. (2010), the dry weight is formed through the assimilation process of CO_2 during plant growth. The root and shoot dry weight of soybean plants was observed 70 days after planting, and the data are presented in Table 3.

The value of dry weight can be used as an indicator of metabolic processes in plants. The higher the dry weight of the plant, the better the plant growth. The root and shoot dry weight tends to increase as the plant ages. Dry weight is an important

Table 3
Dry weight of shoot and root of soybean plants as influenced by NPK fertilizer with diverse filler materials 70 days post-planting

Treatment	Dry weight	
	Root	Shoot
	----- g plant^{-1} -----	
NPK + 10% bentonite clay mineral	3.91 a	52.58 a
NPK + 5% bentonite clay mineral + 5% SBE	4.38 a	60.37 a
NPK + 5% bentonite clay mineral + 5% DBE	4.45 a	61.29 a
CV (%)	15.91	13.37

Note. The same letters that follow the means in the same column do not significantly differ in accordance with the test of least significant difference (LSD) at a 95% confidence interval

observation variable because it shows all processes that occur in the plants. The results indicated that the root and shoot dry weight was not significantly influenced by the three treatments 70 days after planting, which shows that applying SBE and DBE did not influence the two observed variables (Table 3).

CONCLUSION

Overall, the results suggest that the materials of SBE and DBE could partially substitute the filler elements in bentonite clay of NPK fertilizer on a filler basis, which were shown to have the same effect on SOD activity, H_2O_2 content, POD content, MDA, REL, total phenolic content, proline content, and shoot and root dry weight.

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