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Organic Acids from Cocoa Pod Waste Inoculated by Basidiomycota Fungi to Enhance the Performance of Shallots

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ABSTRACT

Excessive agricultural waste potentially causes serious pollution issues by farming practices. The potency of Basidiomycota's fungi to degrade biomass for a safe practice might be considered to cope with this issue. This study aims to determine Basidiomycota fungi's ability to produce organic acids after being inoculated on cocoa husks at various fermentation periods (20 and 40 days) and evaluate the effect of humic acid from those fermented cocoa husks on the growth and productivity of shallots. This research is carried out in three stages; the first is composting process from cocoa husks. The next is the acid-base extraction of cocoa pod husk compost to produce humic and fulvic acid. And the last is the application of humic acid to shallot plants. The results show that composting for 40 days produced higher levels of humic and fulvic acids. Inoculation with *Coprinus sp* produced the highest levels of humic acid but is not different with *Pleurotus sp*. Applying 100 ml/l of humic acid to shallots shows the best growth, while the concentration of 400 ml/l has the best production. This study presents the potential practice of cocoa pod residue and Basidiomycota fungi to increase agricultural commodities' productivity agents.

INTRODUCTION

Organic acids from decomposed soil organic matter enhance the soil fertility that adds important roles in the soil, namely, 1) increasing the acidity or alkalinity of the soil, 2) generating rich nutrients of N, P, K, S, and micronutrients, 3) increasing the ability of the soil to bind and retain nutrients, and 4) affecting the color, texture, and structure of the soil. Rapid depletion of soil organic matter at an annual rate of 5-10% can significantly affect unfertilized crop yields (Sancez, 1993). The contents of soil organic matter (SOM) in Indonesia are very slight, less than 2%. However, the material sources can be obtained by utilizing plant biomass. One of those is cocoa pod husks, whose residue is continually increasing due to the high demand. This by-product has a rich potential source of organic matter to be degraded and used as a natural fertilizer.

The decomposed cocoa pods release various nutrients that play important roles in growth, crop production, and land sustainability. The relationship between organic matter and plant growth can occur directly or indirectly. According to Rao (2010),

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organic matter is a natural substrate for saprophytic microorganisms and indirectly provides nutrients for plants through the activity of microorganisms. Utilization of cocoa husk waste inoculated by the rot fungi *Pleurotus* sp. and *Lycoperdon* sp. from Basidiomycota division on cocoa seedlings show an approximately similar growth when applied with chemical fertilizers. The growth of seedlings applied with chemical fertilizers shows alike the average number of leaves (6.89), leaf area (46.75 cm^2), dry weight of plant (1.53 g), shoot root ratio (3.75), Crop Growth Rate (CGR) (0.69 g/m^2/day), and Net Assimilation Rate (NAR) (0.51 g/m^2/day). Whereas, if it is applied with inoculated-cocoa husk compost with *Lycoperdon* sp. the parameters showed the number of leaves (6.11), leaf area (43.97 cm^2), dry weight of plant (1.56 g), shoot root ratio (4.47), CGR (0.57 g/m^2/day), and NAR (0.51 g/m^2/day) (Asrul, Rahim, Kuswinanti, Rasyid, & Nasruddin, 2018). During decomposition, organic acids such as humic and fulvic acids are produced and play a major role in chemical reactions as a fraction of organic matter.

Black humic acid is often mixed with fertilizers and used in transplant solutions with liquid nitrogen or herbicides. The results showed that this organic acid could retain organic compounds produced from plant decomposition, reduce mineralization, and maintain residual lability in three plant types of vetch > wheat > oak. So, applying humic acid to the residue or unstable litter is highly recommended to reduce mineralization and increase carbon sequestration (Al-Mallahi, Tahhan, & Khresat, 2020)applying humic acid with wheat might have simulated the synthesis of extracellular enzymes and the co-metabolism of humic acid (brimming effect.

Combining humic acid with organic matter can fix nitrogen, reduce leaching and nitrogen loss, and buffer the solution effectively and efficiently, particularly in marginal soils such as acid soils. Adding manure with humic acid also increases plant height, the number of leaves, and the weight of tomatoes in the acid soils. In addition, it also increases the wet weight, dry weight, and root volume of the tomato plant (Rahim, Maharani, Harsani, & Suherman, 2021). Fulvic acid, another extraction of dry humic, can be combined with liquid compounds. Mixing with liquid fertilizer buffer dissolved fertilizer chelate and increased plant absorption. Fulvic acid can also be added with herbicides to increase its effectiveness. It can chelate heavy metals such as Cu and Pb and increase chemical nutrient intake (Mayhew, 2004; Sounthararajah, Loganathan, Kandasamy, & Vigneswaran, 2015). Several reports showed that the mobilization of NPK from the soil to the root system was increased in the availability of humic and fulvic acids. The study of Suntari, Retnowati, Soemarno, & Munir (2015) showed that the application of urea as a source of N combined with humic acid can increase plant height, the number of tillers, and the total dry weight of rice plants by up to 22%. The effectiveness of this nutrient absorption also enhances phosphorus uptake.

Phosphorus deficiency occurs in cultivated plants that grow on soils containing sufficient phosphate because plants only absorb phosphorus in the available active form. According to Rao (2010), available soil phosphate can be absorbed by chelating to organic acids secreted by microbes, such as fungi. Microbes may also release inorganic phosphate dissolved in the soil through decomposition. Adding organic acids also increases the availability of nutrients and the uptake of microelements by plants. Several studies have shown that the application of humic acid increases growth, nutrient uptake, and productivity in crop plants, including spinach (Sarno & Fitria, 2012).

It is quite likely that the rot fungi will be used to degrade cocoa pods husk into organic acids. This study used the acid-base extraction technique to separate humic and fulvic acids from cocoa pod husks that had been inoculated with several basidiomycetes fungi. This research aims to determine the best isolate of fungus in generating organic acids and evaluate the effect of humic acid on shallot growth performance.

MATERIALS AND METHODS

Extraction of humic and fulvic acids was carried out at the Laboratory of Biotechnology and Tree Breeding, Faculty of Forestry, Hasanuddin University, Makassar, South Sulawesi, in May 2021. Humic acid was applied in shallot plants at the screenhouse in Universitas Muhammadiyah Parepare from June to August 2021.

Composting Process of Cocoa Husk

Cocoa husks have been stored for 30 days, air-dried, and chopped into 1-2 cm. The pod husk was then mixed with rice hulls and bran with a ratio of 70:25:5. The mixture was treated with inoculants of *Mycena* sp., *Lycoperdon* sp., *Coprinus* sp., *Tremella* sp., and *Pleurotus* sp. that had been grown on Potato Dextrose Broth. Another treatment was free inoculant as a control. Compost was mixed with rot fungi, then added to water until it reached a concentration of 30%, and fermented for 60 days. The fungi with the highest ability to produce humic acid were applied to shallot plants.

Scanning Electron Microscope (SEM) of Rot Fungi-Compost

The ripe compost of cocoa husks was scanned with ultrastructural micrographs (Koga, Kusumi, Shodo, Dan, & Ushiki, 2015) using SEM Hitachi SU 3500. Several pieces of compost were dissected into a size of 5 x 5 mm2, washed with distilled water, then contrasted with 1% uranyl acetate for 10 minutes and 1% lead solution in citrate water for 5 minutes. The setting was placed on aluminum stubs using carbon tape, metal-coated, then examined under SEM operating at 10 kV. The results of the electron micrograph highlighted the microscopic structure of the substrate.

Extraction of Humic Acid and Fulvic Acid from Cocoa Husk Compost

The concentrations of humic and fulvic acids were determined using the method of acid-base extraction (Tan, 1991). About 10 g of cocoa husk compost was added with 50 ml of 0.1 N NaOH and then shaken for 24 hours. After that, 3 ml of saturated NaCl was added, left overnight, then filtered using Whatman 42 filter paper to obtain the filtrates. Humic acid was extracted by adding a few drops of concentrated HCl when the solution pH was adjusted to one, then heated in a water bath at 80 ^{o}C for 30 minutes. The filtrate was left for 24 hours and then filtered again. Humate is a nonsoluble fraction in water, so it remains on the filter paper, such as black extract precipitate. Fulvate is soluble and thus filtered as a yellow extract. The humic fraction was roasted for 48 hours to attain the dry weight, while the absorbance of fulvic acid was measured with a wavelength of 561 nm.

The humic acid concentration was measured using a standard curve with the regression equation of $Y = 0.1319 x + 0.1384$, with $r = 0.9672$ (Dahniarti, Destiarti, & Idiawati, 2016). Equations were obtained from series dilutions of 2, 3, 4, 5, and 6 ppm, added with 2.5 ml of 0.1 M NaOH solution. The absorbance was measured at a wavelength of λ 200-800 nm using a UV-VIS spectrophotometer and then compared with standard curves.

Application of Humic Acid on Shallot Plants

The stock solution was made by dissolving 1 ml of pure humate from the extraction of cocoa husk compost in one liter of distilled water. The concentration of humic acid from 20 days and 40 days fermentation periods is divided into four treatments, each as follows: H0 = water without humic acid, H1 = 100 ml of humic acid per liter of water, H2 = 200 ml/l, M3 = 300 ml/l, and H4 = 400 ml/l. The treatments were arranged using a Randomized Block Design with three replications. The application of humic acid was carried out by spraying the solution throughout the whole plant every week until the harvest period. Data were analyzed using covariance analysis to see the significance of humic acid on shallot.

RESULTS AND DISCUSSION

The Microscopic Structure of Compost with SEM

The microscopic structure of scanned cacao pods compost shows a cross-sectional view (Fig. 1). The parenchyma tissue is seen to be occupied by large pores. Fungal mycelia are detected very clearly and scattered in the vascular fibers. Inoculation using *Pleurotus* sp., a white rot fungus, degrades the cocoa pod husk into smaller flakes. Moskal-del Hoyo, Wachowiak, & Blanchette (2010) described that infection with rot fungi resulted from simultaneously degrading the whole cell wall components. It was characterized by a spongy appearance and extensive mycelial growth that invaded most tissues. *Pleurotus* sp. decomposes carbohydrates and lignin at the same rate and simultaneously during all stages of decomposition. Cell wall decay begins with microhyphae holes in the secondary wall, which flow together to form larger wall openings as decay progresses. Hyphae thrive in the lumen near the tertiary boundary. The hyphae are surrounded by a layer of mucus, which secretes the active substance when it comes into contact with it. As a result, a zone of hyphal lysis develops beneath, and the hyphae produce grooves in the wall that gradually decreases in thickness as the soil is eroded by a river (Schmidt, 2006). Organic matter will gradually be destroyed and decomposed. A study by Fadzilah, Saini, & Atong (2015) on palm frond compost inoculated with rot fungi *Tremetes* sp. also showed scattered mycelium on the surface and vascular tissue with silica deposited in circular craters with perforated bottoms.

Measurement of Humic Acid and Fulvic Acid Concentrations

White rot fungi synthesize organic acids from their biological activities of organic matter degradation. Fulvic acid is extracted by separating humic acid using a strong acid solution. Humic acid was found in the precipitate fraction, while fulvic acid was found as a liquid. Inoculation using Pleurotus sp on cocoa husks showed that the highest humic acid concentration was produced in the long-fermented storage. It is indicated by the density of the supernatant solution resulting from acid-base extraction. Fermentation for 40 days shows a concentrated supernatant (Fig. 2).

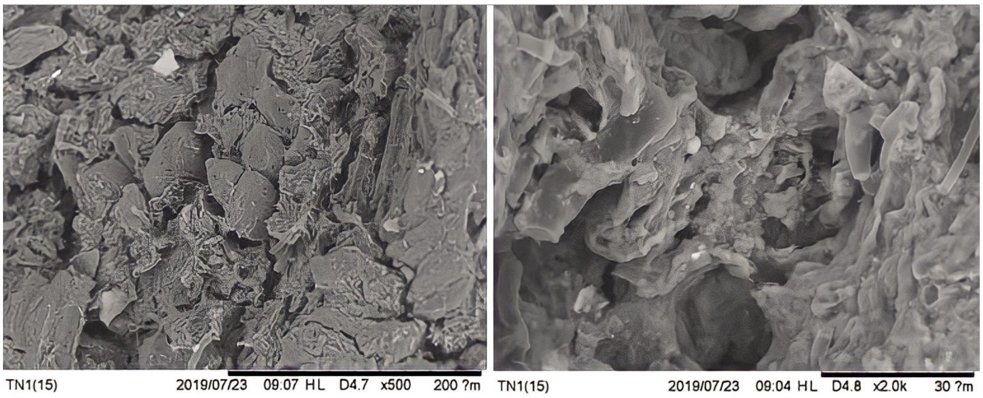

Fig. 1. Ultrastructure electron micrograph of cocoa pod waste compost inoculated with *Pleurotus* sp. 9

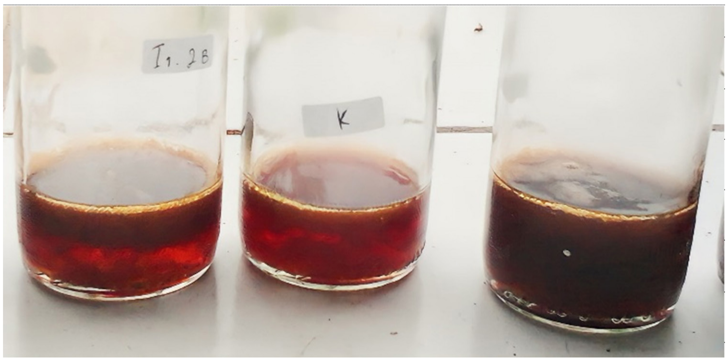

Fig. 2. Extraction of cocoa pod husk compost. The longer the fermentation storage, the dense color of the liquid. Fermentation without fungi isolates (K), 20 days of fermentation (A), and 40 days of fermentation periods with *Pleurotus* sp. (B)

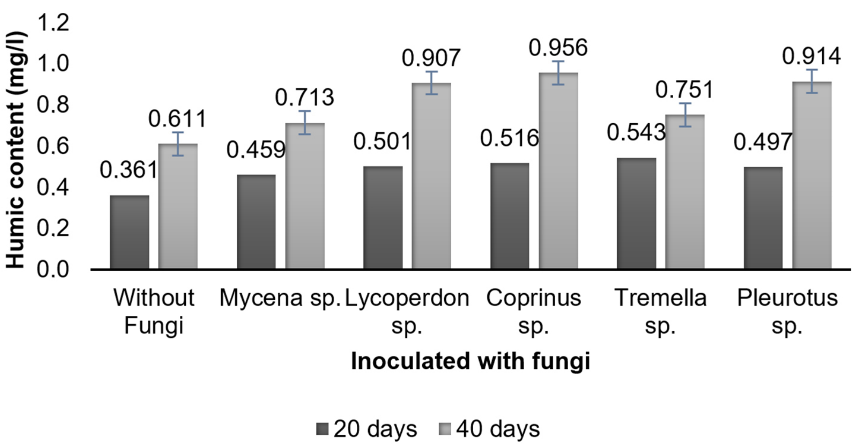

Fig. 3. The humic acid contents of cocoa pod husk compost inoculated with various rot white fungi

The highest humic acid concentration at 40 days of fermentation periods was inoculated with *Coprinus* sp., followed by *Pleurotus* sp.(Fig. 3). The lowest content is in free rot fungi fermentation at 20 and 40 days. This study also finds that rot fungi inoculation on cocoa pod husk waste increases the humic acid contents. This organic acid is available in the form of salts with inorganic mineral elements. Analysis of natural extracts shows the presence of 60 mineral elements. A wide variety of living organisms readily utilize these humic-chelated elements. As a result, humic acids play an essential role in ion exchange and metal/chelating complexation systems (Pettit, 2004).

Composting storage resulted in different humic acid contents. At 20 days of fermentation, the percentage of humic acid ranged from 0.361-0.543 µg/l and increased twice to 0.611-0.956 µg/l at the 40 days. He, Traina, & Logan (1992) stated that the duration of composting will determine the level of mature compost. The contents of fulvic acid indicate this maturity. At the initial stage of decomposing organic matter, fulvic acid is somewhat richer produced than humic acid. But when the degradation process proceeds, the fulvic acid decreases or remains constant while the humic acid tends to increase (Inbar, Chen, & Hadar, 1990). Agustian, Susila, & Gusnidar (2004) also showed that the humic acid content of rice straw compost increased from 16.62 mg/g at 20 days to 20.87 mg/g at 40 days of fermentation after being applied with 10 ml of EM4. This value is higher than the humic acid content presented in this study with similar days of fermentation periods. The different material sources might cause this. However, several vital factors affect the properties and characteristics of the produced compost. The composition of organic matter will determine the convenient degradation (He, Traina, & Logan, 1992).

Humus consists of three major fractions defined as humic acid, fulvic acid, and humin from a reaction of humus complex with solvent extraction. Humic acid makes up the bulk of the humus complex and is considered a polymer of aromatic compounds. Fulvic acid is the soluble fraction of soil organic matter, which is acidic or alkalis and contains carbohydrates and proteins (Rao, 2010). The content of fulvic acid is indicated by the purity and turbidity of the organic matter solution. The dense solution exhibits higher absorbance, which indicates a higher concentration of fulvic acid. This concentration increased with increasing fermentation durations (Table 1).

The absorbance of humic acid at the 20 days ranges from 0.186 – 0.211, while at the 40 days, it ranges from 0.219 – 0.265 (Tabel 1). The highest fulvic acid content is presented in *Coprinus* sp. isolates, and the lowest was in the organic matter without isolates. There is also a significant difference in the wet and dry weight of the cocoa husk compost deposit between 40 and 20 days of fermentation. The precipitate extracts contained the amount of humic acid in 10 g of compost. The highest weight of humic acid is shown in *Mycena* sp. isolate, and the lowest was in *Pleurotus* sp. isolate, which was 3.975 g and 3.745 g. The Mycena genus has a distinctive active substance: the antifungal compound strirobilun and or oudemansins. These compounds give the species a competitive advantage over potential competitors. It can be one of the causes of the high humic acid produced by giving Mycena sp. (Aqueveque et al., 2005). However, *Pleurotus* sp. has the highest dry weight compared to other fungi. It shows that *Mycena* sp. has a higher ability to absorb water, while *Pleurotus* sp. has much mycelium that might increase the dry weight. These findings lead to the identification of superior fungi for humic acid extraction from organic matter. *P. ostreatus* is a basidiomycete that produces laccase as the main ligninolytic enzyme. These available carbohydrates in lignocellulosic waste support biomass formation and enzyme production by ligninolytic fungi (Das, Bhattacharya, Panchanan, Navya, & Nambiar, 2016). Research by Alemawor, Dzogbefia, Oddoye, & Oldham (2009) shows an increase in the composition of fibrous cocoa pods as a result of fermentation with *P. ostreatus*. Pleurotus species also showed intense ligninolytic activity on lignocellulosic residues, including sugarcane residues.

This ligninolytic activity indicates the ability of the fungus to decompose lignin and cellulose, which are constituents of organic matter. This decomposition causes the existing nutrient content to be released from the body of organic matter. It is indicated by the high nutrient content of the compost with *Pleurotus* sp. (Table 2).

Table 2 shows that the application of rotting fungi had a significant effect on the nutrient content of the cocoa pod compost. The highest nutrient levels of total N, P, K, organic C, and Mg were obtained in compost fermented with *Pleurotus* sp. The composting process is highly dependent on the activity of reorganizing microorganisms. Microorganisms need a source of C to get energy and N as a material for the formation and multiplication of cells. Without a source of C in the form of simple sugars and good N, the breakdown process will take place slowly. Microorganisms need nitrogen for the synthesis of protoplasm. If N is unavailable, it will reduce the ability of fungi to break down carbohydrates.

Meanwhile, plants need N as a form of amino acids, enzymes, proteins, and nucleic acids (Fisher & Binkley, 2000). Meanwhile, the magnesium produced is a released nutrient. Microbes in the mineralization process other than N, P, K, and Ca. Mg is an essential constituent of chlorophyll and functions as a cofactor in enzymes. It directs *Pleurotus* sp. to select superior fungi for humic acid extraction on organic matter.

Application of Humic Acid on Onion Plants

The application of humic acid on shallot plants showed better growth than without humic acid, although the direct performance did not show much difference (Fig. 4). These results aligned with the greenhouse experiment by Cristina et al. (2020) using a Chilean lettuce plant (*Lactuca sativa* L.) treated with alginic-humic acid extract beads. The hypogean dry biomass from the treated lettuce was significantly higher than those of the untreated plants. Likewise, a study on barley plants showed that plant height, nail length, grain yield, straw, and 1000-grain weight increased significantly with the application of humic acid (Belal, El Sowfy, & Rady, 2019). Humic acid can increase the chlorophyll content and the uptake of nutrients by roots to shoots of long beans. In general, humic acid increases peanut yield and tolerance (Li et al., 2021), wheat grain yield (ul Hussan et al., 2022), and leaf growth of *Stevia rebaudiana*, particularly under drought stress (Zamani, Karimi, Abbasi-surki, & Direkvand-moghadam, 2021).

Humic acid is an organic compound that has undergone a humification process and is soluble in alkali. Humic acids have a direct and indirect effect. It indirectly improves soil fertility status regarding soil's physical, chemical, and biological properties (Tan, 1991). It increases soil fertility status and the uptake of plant nutrients, thus maintaining and increasing plant growth and productivity. In direct effect, humic acid improves a plant's metabolic processes, such as increasing the rate of plant photosynthesis (Heil, 2005), following the increased chlorophyll content in the leaves (Ferrara & Brunetti, 2010). However, the exact concentration of humic acid for growth and production in shallot plants is poorly explored. This study provides the effect of humic acid with various concentrations on shallot plants (Table 3).

Table 1. The absorbance of fulvic acid, dry weight, and wet weight of white-rot fungi from acid-base extraction

Fungi	Day 20 of fermentation			Day 40 of fermentation		
	Absorbance of fulvic acid	Wet Weight (g)	Dry Weight (g)	Absorbance of fulvic acid	Wet Weight (g)	Dry Weight (g)
Without Fungi	0.186	1.692	0.028	0.219	2.930	0.048
Mycena sp.	0.199	1.719	0.036	0.233	3.975	0.103
Lycoperdon sp.	0.205	1.797	0.034	0.258	3.818	0.105
Coprinus sp.	0.207	1.804	0.034	0.265	3.830	0.105
Tremella sp.	0.204	1.583	0.029	0.259	3.425	0.101
Pleurotus sp.	0.211	1.515	0.031	0.227	3.747	0.133

Table 2. utrient content of cocoa pod skin compost by rotting fungus fermentation

Compost with Rot fungi	N Total	P_2O_5	K_2O	C Organik	Mg
	-- % --				
Without fungi	0.395 a	3.230 a	0.264 a	6.250 a	0.103 a
Mycena sp.	0.448 a	4.255 ab	0.358 ab	11.125 b	0.160 ab
Lycopercon sp.	0.520 a	4.558 ab	0.405 b	12.605 b	0.158 ab
Coprinus sp.	0.490 a	5.140 ab	0.445 b	12.620 b	0.175 ab
Tremella sp.	0.513 a	5.055 ab	0.523 bc	14.243 b	0.170 ab
***Pleurotus* sp.**	**0.615 b**	**5.948 b**	**0.620 c**	**16.668 b**	**0.250 b**

The growth of shallot plants at various levels of humic acid showed no significant difference between concentrations, but generally, there were differences compared to controls. The humic acid concentration of 100 ml/l showed a relatively optimal growth compared to other treatments, particularly for leaf number, plant height, and biomass. The application of humic acid also positively affected root parameters of root length and weight, which was the highest at a concentration of 400 ml/l. In contrast, the highest production of shallots was shown with humic acid treatment at 300 ml/l, namely the number of tubers, diameter, and weight. It followed the shallots' research on water-deficiency lands where humic acid was applied. Humic acid significantly increased growth and quality properties, including leaf biomass, vitamin C, and minerals such as Ca, as well as tuber flavonoids under water deficit stress (Forotaghe, Souri, Jahromi, & Torkashvand, 2022).

The application of humic acid to other horticultural commodities, namely chili, with a dose of 50 g/l resulted in the maximum number of leaves, plant branches, plant height, stem diameter, number of leaves, and fruit, and the productivity reached 3.93 t/ha. The 100 g/l humic acid concentration was the best dose for the chili plant to induce a short flowering period, fruit weight, diameter, and volume (Jan et al., 2020). The combination of 60 ml/l of humic acids with *Azotobacter chroococcum* increased guava (*Psidium guajava*) fruit production by 1.89 times compared to the control (Ashwini et al., 2022).

Fig. 4. Performance of shallots applied with various concentrations of humic acid from the extract of cocoa pod husk compost

Table 3. Plant growth of shallot applied with humic acid from cocoa pod husk compost inoculated with rot fungi

Level of humic acids (ml/l)	Parameter per plant							
	Number of leaves (strands)	Plant height (cm)	Plant biomass (g)	Root length (cm)	Root weight (g)	Number of tubers	Diameter of tubers (mm)	Bulb weight (g)
0	40.00	39.01	55.00	16.53	0.47	8.20	2.13	535.00
100	43.27	39.10	56.00	19.01	0.44	8.47	1.80	576.00
200	38.20	38.99	56.27	16.98	0.35	8.87	2.16	535.00
300	40.07	39.47	46.60	17.61	0.54	9.80	2.19	582.00
400	44.13	39.38	54.73	19.93	0.56	7.53	1.84	563.00

Organic matter content and balanced nutrient concentrations are associated with high soil fertility and structure. Humic acid contains nutrients that boost soil fertility, increase nutrient availability, mitigate the negative effects of chemical fertilizers, and remove toxic NO_2 and NO_3 ions from the soil. It causes humic acid to improve plant performance (Osman & Rady, 2012). Humate is the main component of organic matter in the soil (Sangeeetha, 2006), and it can improve soil fertility by changing the chemical and biological conditions in the soil. It can also increase the biomass and performance of plants. Humic substances, including humic acid, impact plant performance directly and indirectly (Desoky, Merwad, & Rady, 2018; Nardi, Pizzeghello, Muscolo, & Vianello, 2002). Humic substances can indirectly improve soil properties and structures.

These results indicated that using humic acid can increase the growth and production of agricultural commodities. Humic acid can be obtained from extracting organic material from agricultural waste, especially cocoa pod husks that have not been used, to support the sustainability of eco-friendly agriculture.

CONCLUSION

Fermentation with *Coprinus* sp. and *Pleurotus* sp. for 40 days resulted in higher humic acid content reaching 0.956 g/l and 0.914 g/l, respectively. Spraying 400 ml/l of humic acid to shallots increased root length and weight, while a concentration of 300 ml/l of humic acid showed higher production of tubers. In the forthcoming study, observation of organic acid concentrations from other abundant agricultural biomass, such as empty fruit bunches of oil palm, can be compared using a similar method.

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