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Cover Photo: Female Gayo Horse (photo taken by Juli Melia, the first author of the manuscript titled "Biological Characteristics of Indonesian Gayo Horse", published in this issue of BIOTROPIA Journal)

BIOLOGICAL CHARACTERISTICS OF INDONESIAN GAYO HORSE

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ABSTRACT

This study aimed to find out the characteristics of Gayo horses and to identify the population of Gayo horses. Data collection was conducted through a selection process from more than 100 local horses distributed in Central Aceh Subdistricts, Gayo Lues and Bener Meriah. The selection process resulted in 30 Gayo horses having varying ages. Detail observation was performed on six Gayo horses consisting of 3 male horses and 3 female horses. The observation comprised morphological observation and morphometry. Data collection of Gayo horse population was based on the annual report from the local Animal Husbandry and Fishery Office of Central Aceh Subdistrict over the last 5 years. Results of this study showed that Gayo horses have a straight *cranial* shape with smaller size, thick and stiff mane, and ears akin to donkey's ears. The study also showed that Gayo horse's body height ranged between 113-120 cm with a body weight range of 215-280 kg. Gayo horse is agile despite living in mountainous areas and able to carry heavy loads. The population of Gayo horses have been declining since 2010-2014. Gayo horse is included in a large pony category. Conservation efforts are essential to save Gayo horse from extinction.

Keywords: characteristics, Gayo horse, hallmarks, population

INTRODUCTION

Indonesian local horses are among livestock commodities supporting national development, especially in the animal husbandry subsector. Gayo horse is one of the Indonesian native horse germplasms (Soehardjono 1990). This has been written in the Ministry of Agriculture Decree No. 1054/Kpts/SR.120/10/2014 which stated that the Gayo horse is an Indonesian native horse strain.

Gayo horse population is spread all over Gayo highland in the Central Aceh Subdistrict, including its autonomous regions called Gayo Lues and Bener Meriah. Central Aceh Subdistrict is known by various other names such as "Nation Above the Clouds", "Gayo Highland" and "*Negeri Antara*". In this region, the annual Gayo traditional horse race was held to commemorate the Proclamation of Independence of the Republic of Indonesia and the anniversary of the Central Aceh Subdistrict. Many owners of Gayo horses have the intention to breed Gayo horse with a thoroughbred horse, creating a new type of horse known as "Astaga horse" (Australia-Gayo). Nowadays, pure Gayo horse is hard to find, which indicated that there might have been a very drastic decline of the Gayo horse population. The attempt to conserve the Gayo horse as one of the Indonesian native horse germplasms must be done immediately to save the Gayo horse from extinction.

The objective of this study is to describe Gayo horses' characteristics and to identify the number of Gayo horses' population, which is crucial for future research concerning the reproduction of Gayo horses.

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MATERIALS AND METHODS

Research Procedure

Data collection was carried out by means of direct identification at the Gayo horses' habitat. Additionally, the past and present information on Gayo horses was obtained through direct

interviews with several owners of Gayo horses and with officials of the local Animal Husbandry and Fisheries Office of the Central Aceh Subdistrict. One hundred local horses were selected from areas surrounding the Central Aceh Subdistrict and its autonomous regions called Gayo Lues and Bener Meriah. In the initial phase, the identification process was conducted according to the criteria written in the Ministry of Agriculture Decree No. 1054/Kpts/SR.120/10/2014. Of the 100 selected local horses, 30 horses were identified as Gayo horses having varying ages. A more thorough identification process was conducted on 6 Gayo horses consisting of 3 male horses and 3 female horses.

Gayo Horse Morphology and Morphometry

The morphological examination was carried out visually and documented by SLR camera (Nikon D3200, AF-S DX Zoom-Nikkor 18-55mmf/3,5-5,6G ED II). The morphological examination included observation on body shape, eye shape, mane condition, head shape, ear shape, neck shape, nape and tail. Morphometry examination included measurement on body height, girth and body length. Body height was measured from the tip of the front leg up to its withers. The girth was measured from the lower abdomen from the *fossa olecranon* perpendicular with *os sternum* until above *os vertebrae*. Body length was measured from the connection of *cartilago* and *os scapula* horizontally until behind the horse's *os pelvis*. Body weight measurement is predicted by using the Schoorl formula: (Body weight = (chest circumference + 22)^2/100) and measured in kilograms (kg).

Population Data

Data on Gayo horses' population were collected from the annual reports of the years 2010-2014 published by the local Animal Husbandry and Fisheries Office of Central Aceh Subdistrict and its autonomous regions, i.e., Gayo Lues and Bener Meriah.

Data Analysis

Gayo horses' morphology and morphometry data were descriptively analyzed. The population data were analyzed and depicted in graphs. The prediction of Gayo horses' population extinction was analyzed exponentially by using Excel 2016 software.

RESULTS AND DISCUSSION

Gayo horses are included into large ponies for having a body height of not more than 1.47 m. On the other hand, miniature ponies have body height ranging from ≤ 86 cm (A type) up to 88.6 cm (B type) (Campbell 1992). According to Ensminger (1962), a pony's height is less than 1.45 m while standing, with 250-450 kg body weight, and usually are descendants of lightweight horses.

Being included as large ponies, Gayo horses have different body sizes compared to foreign horses. The size differences are often influenced by environmental factors, such as topography and climate (Ohsawa *et al.* 2008; Steinheim *et al.* 2008; Kosoma & Purzyc 2009). Ponies are often used for recreation purposes (Rogers *et al.* 2006), and for therapy programs (Burke 2002).

Generally, Gayo horses have similar characteristics to other local horse types in Indonesia. However, Gayo horses have several uniqueness, such as having straight head shape with smaller body size, thick and stiff mane, and having ears similar to donkey's ears (Table 1; Fig. 1).

Table 1 The morphology of Gayo horses

Morphology	Shape	Color
Body	Short	Black, dun, chestnut, grey, white
Eye	Small	Black
Gaze	Sharp	
Mane	Thick and stiff	
Head	Straight	
Ear	Similar to donkey	
Nape	Long	
Throatlatch	Wide	
Tail	Medium to long	Similar to body color, mix

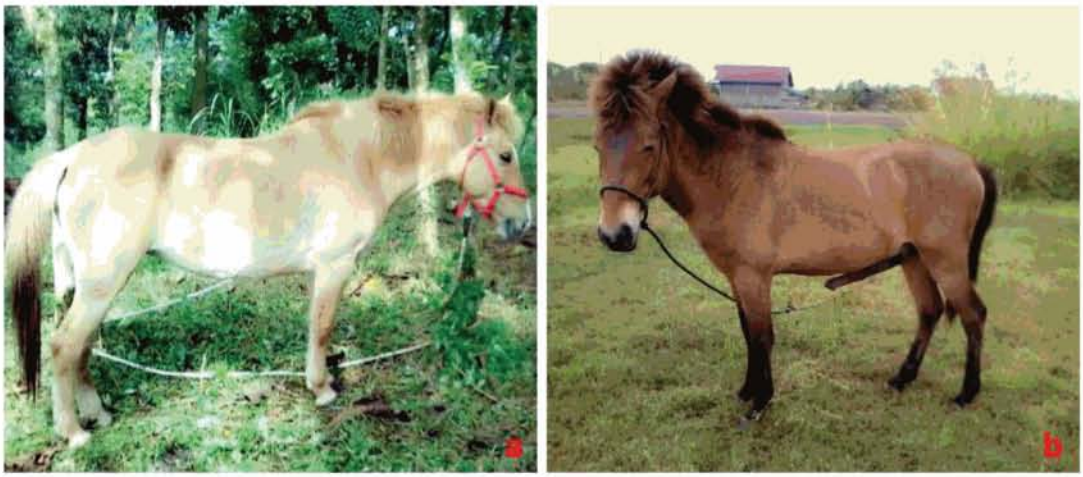

Figure 1 Gayo horse appearance
Notes: a. female; b. male.

Gayo horses have body height of 113-120 cm, chest circumference of 136-139 cm, body length of 102-105 cm, and body weight of 215-280 kg (Table 2). A pictured guide for easy identification of Gayo horse in the field is presented in Figure 2.

Table 2 The morphometry of Gayo horses

Morphometry	Height (cm)	Chest circumference (cm)	Body length (cm)	Weight (kg)
Male	118-120 (119.00±1.00)	137-139 (138.00±1.00)	103-105 (104.33±1.15)	225-280 (256.67±28.43)
Female	113-115 (114.33±1.15)	136-138 (137.00±1.00)	102-105 (103.67±1.53)	215-250 (233.33±17.56)

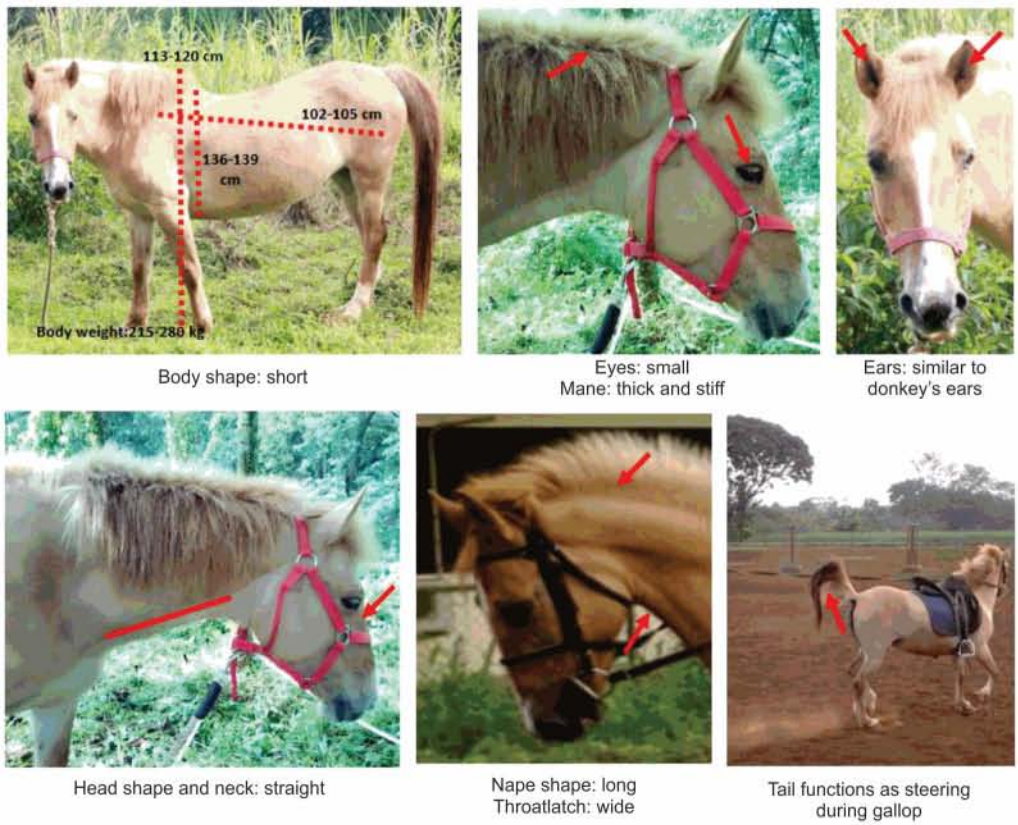

Figure 2 A pictured guide for easy identification of Gayo horses in the field

Several distinctive marks indicating the temperament and intelligence of local Indonesian horses, which also exist in Gayo horses, are the condition and shape of hair whorl, i.e., 1. *Pusar cekak*; hair whorl under the jaw, indicating that the horse is always being preyed upon by tiger; 2. *Pusar terbang*; hair whorl by the front feet around the left and right knees area, indicating that the horse is suitable as a racehorse due to its fast speed; 3. *Pusar dada*; hair whorl by the chest, indicating that the horse can spin fast and not fall in a sharp turn during a race; 4. *Pusar gedung*; hair whorl by the flank or below the flank, indicating a mild or tame temperament and is suitable as brood; 5. *Pusar turun tangis*; hair whorl under both eyes, indicating temperamental personality, sometimes being tame and sometimes being wild; 6. *Pusar ruk*; hair whorl by the hind legs behind the knees, indicating a wild and hard to control personality; 7. *Pusar lipan*; hair whorl around the throat and under the ear, indicating a hard to control or wild personality.

Gayo horses have several positive characteristics, such as power and agility, having capabilities to carry heavy loads, having good temperament, having good endurance, having high adaptivity toward various environmental conditions, having survival capabilities with minimal feed availability, and having easy maintenance, which makes Gayo horses very economical for its owner. Judging from various aspects, Gayo horses have considerably high strategic values. Based on cultural aspects, the Gayo horse symbolizes social status. Usually, a boy's manhood is determined from the moment a boy can ride a Gayo horse without a saddle in a race, which custom is still practiced nowadays. From an economical aspect, the Gayo horse is used as a source of animal protein for the local people who consume horse meat. Gayo horse is often sold to other regions such as North Sumatra to be consumed. From the utilization aspect, the Gayo horse is used as a plow puller in rice fields and as a freight horse to transport agricultural products. Based on data obtained from the local Animal Husbandry and Fisheries Office of the Central Aceh Subdistrict, the population of Gayo horses drastically decrease in 2010-2014 (Fig. 3).

Prediction analysis indicated that Gayo horse will extinct in 2037 (Fig. 4). Main reasons that might cause the decrease of the Gayo horse population: 1. Crossbreeding; 2. Uncontrolled culling; 3. Existence of modern machinery.

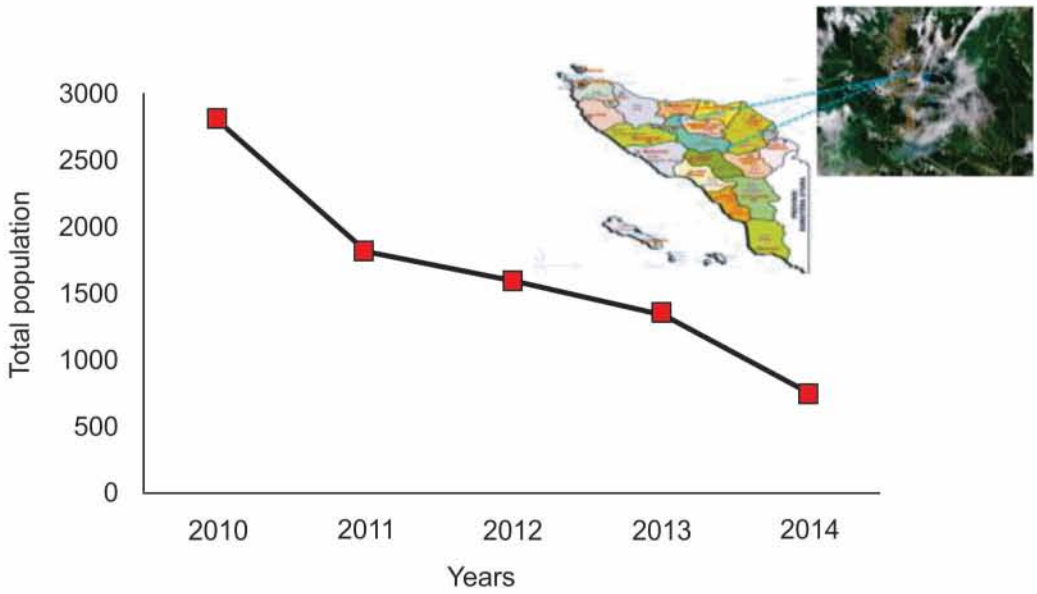

Figure 3 Gayo horses population at Central Aceh Subdistrict

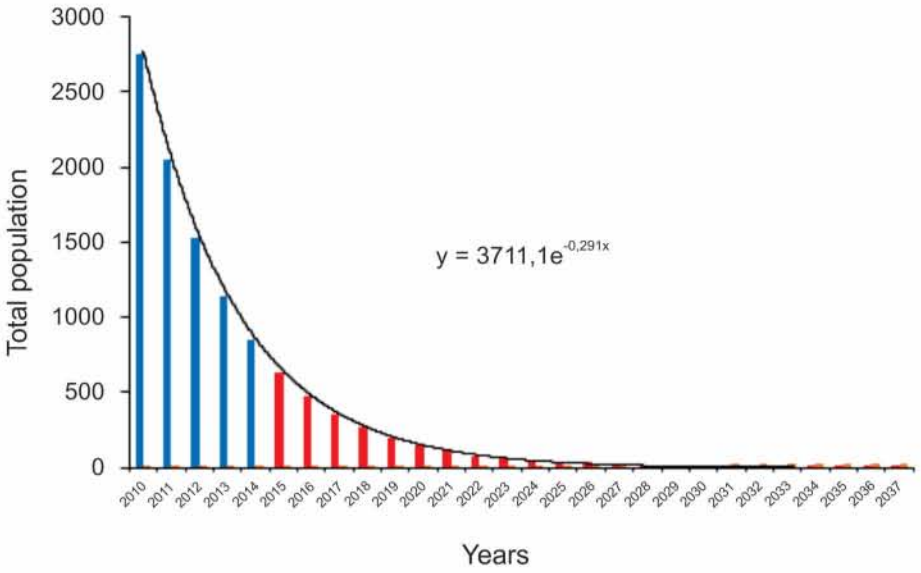

Figure 4 Prediction of Gayo horse extinction

The upkeeping of Gayo horses is usually done conventionally. After the agricultural harvest season, the Gayo horses are released to the mountainous area and rice fields. The mating system of the Gayo horse is akin to that of primates. Only superior stallions may mate with the female horses around the area where the stallion is kept. If a stallion is defeated in a fight before mating, the defeated stallion will be moved to the other group of horses, which causes inbreeding leading to the decrease of body size, height, and weight of Gayo horses. Nowadays, the upkeeping system of Gayo horses has separated the stallion from the mare Gayo horses, even though it is still conventionally conducted. The mare horse is brought to the stallion only at the breeding time.

The body maturity of Gayo horses occurs at 12-15 months of age, while sexual maturity starts at 12-18 months of age (Personal communication with owners). Usually, the mare of the Gayo horse has 21 days of the estrus cycle. All information obtained from owners was proven through complete research on the reproduction status of Gayo horses. Determination of reproduction status is among the most important factor of animal nurturing and breeding management, including for horses, because reproduction status has a close link with animal's basic reproductive physiology. A number of techniques can be utilized, such as ovarium dynamic observation by using ultrasonography (Amrozi *et al.* 2004; Cuervo-Arango & Newcombe 2008; Derar & Hussein 2011; Melia *et al.* 2014), hormonal analysis (Agil *et al.* 2008) and histology examination on reproductive organs. Hormonal analysis in a horse is generally conducted using the horse's blood samples (Bollwein *et al.* 2002; Utt *et al.* 2007; Ginther *et al.* 2010). All of these data can be used as the basic reproductive physiology data of Gayo horses, which will support the application of reproductive technology as one of the means to save endangered the population of Gayo horses from extinction.

CONCLUSION

Gayo horse is included in a large pony category. Conservation efforts are essential to save Gayo horses from extinction.

ACKNOWLEDGMENTS

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REFERENCES

Agil M, Supriatna I, Purwantara B, Candra D. 2008. Assessment of Fertility Status in the Male Sumatran Rhino at the Sumatran Rhino Sanctuary, Way Kambas National Park, Lampung. HAYATI Journal of Bioscience 15:39-48.

Amrozi, Kamimura S, Ando T, Hamana K. 2004. Distribution of estrogen receptor alpha in the dominant follicles and corpus luteum at the three stages of the estrous cycle in Japanese black cows. J Vet Med Sci 66(10):1183-8.

Bollwein H, Mayer R, Weber F, Stolla R. 2002. Luteal blood flow during the estrous cycle in mares. Theriogenology 65:2043-51.

Burke D. 2002. Australian Miniature ponies. [accessed 2013 April 06]. Available at: http://burkesbackyard.com.au/2002/archives/2002/road tests/horse_breeds/australian_miniature_ponies

Campbell ME. 1992. Selected aspects of miniature horse reproduction. Proceeding of the Annual Meeting of the Society for Theriogenology. p. 89-96.

Cuervo-Arango J Newcombe JR. 2008. Repeatability of preovulatory follicular diameter and uterine edema pattern in two consecutive cycles in the mare and how they are influenced by ovulation inductors. Theriogenology 69:681-7.

Ensminger ME. 1962. Animal Science. Animal Agriculture Series. 5th Ed. Danville (US): Printers & Publishers. Inc.

Derar RI, Hussein HA. 2011. Ovarian Follicular Dynamics during the Estrous Cycle in Jennies in Upper Egypt. Veterinary Medicine International Article ID 860518, 6 p. https://doi:10.4061/ 2011 /860518.

Ginther OJ, Almamun, Shahiduzzaman AKM, Beg MA. 2010. Disruption of the periovulatory LH surge by a transient increase in circulating 17β-estradiol at the time of ovulation in mares. J Anim R Sci 117:178-82.

Kosoma M, Purzyc H. 2009. Konik and Hucul Horses: A comparative study of exterior measurements. J Anim Sci 87:2245-54.

Melia J, Amrozi, Tumbelaka LI. 2014. Ovarian Dynamics of Endometritis Cows Treated with a Combination of Intrauterine Infusion of Gentamicin, Flumequine, and PGF2α Analog. J Ked Hewan 8(2):111-5.

Ohsawa T, Saito Y, Sawada H, Ide Y. 2008. Impact of altitude and topography on the genetic diversity of *Quercus serrata* populations in the Chichibu Mountain central Japan. J Anim Sci 203(3):187-96.

Rogers CW, Gee EK, Hangoor E, Firth EC. 2006. Preliminary survey of congenital and reproductive disorders in the New Zealand Miniature horse population. Proceedings of the New Zealand Society of Animal Production 66:274-8.

Steinheim G, Odegard J, Ådnoy T, Klemetsdal G. 2008. Genotype by environment interaction for lamb weaning weight in two Norwegian Sheep breeds. J Anim Sci 86:33-9.

Soehardjono O. 1990. Kuda. Jakarta (ID): Yayasan Pamulang.

Utt MD, Acosta TJ, Wiltbank MC, Ginther OJ. 2007. Acute effects of prostaglandin $F_{2\alpha}$ on systemic oxytocin and progesterone concentrations during the mid-or late-luteal phase in mares. J Anim R Sci 97:63-73.

THE RANGE EXPANSION OF *Parachromis managuensis* GÜNTHER, 1867 (PERCIFORMES, CICHLIDAE) IN JAVA, INDONESIA

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ABSTRACT

The Jaguar cichlid, *Parachromis managuensis* (Günther, 1867), is native to Central America, with introductions reported from West Java and Central Java Provinces of Indonesia. On 7-8 January 2019, sixteen specimens of *P. managuensis* were collected from Karangkates, the largest hydropower reservoir in East Java Province of Indonesia. A description of the morphological characters of specimens is provided.

Keywords: Cichlid, distribution, freshwater fish, Jaguar Guapote

INTRODUCTION

Parachromis managuensis (Günther, 1867), is a cichlid native to Costa Rica, Nicaragua and Honduras (Conkel 1993), but it has been introduced to several countries in North America (Fuller *et al.* 1999), South America (Magalhães & Jacobi 2013), and Southeast Asia (Agasen *et al.* 2006). *Parachromis managuensis* exhibits highly predatory habits and tolerance to new habitats (Rosana *et al.* 2006; Agasen *et al.* 2006), which make *P. managuensis* potential to become an invasive species (Yamamota & Annete 2000).

Parachromis managuensis is generally sold as ornamental fish and has not been cultured openly. *P. managuensis* in Indonesia is firstly found in the freshwaters of West Java (Dahruddin *et al.* 2016) and Central Java (Hedianto *et al.* 2013) Provinces of Indonesia. The presence of *P. managuensis* at the Karangkates Reservoir is considered a new finding because there has been no previous record of exotic fish culture in Karangkates Reservoir, the largest hydropower reservoir in East Java Province.

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MATERIALS AND METHODS

The Fish Sampling and Description of the Study Sites

Sixteen (16) live specimens of *P. managuensis* were obtained from a local angler during fieldwork conducted on 7 - 8 January 2019 at the Karangkates Reservoir (8°11'16"S; 112°27'22"E) (Fig. 1). Administratively, the Karangkates Reservoir is located in Malang Regency, East Java Province, Indonesia. Fishing gear used by the angler was a medium hook with a bottom and using worms as bait (Stein *et al.* 2012).

Figure 1 The collecting point of *Parachromis managuensis* at the Karangkates Reservoir in East Java Province

Fish Identification

The morphological characters of the fish specimens were determined by using the methods employed by Kullander and Hartel (1997) and Bussing (1998).

RESULTS AND DISCUSSION

Specimens Collection

The sixteen (16) live specimens of *P. managuensis* had a range of total length between 9.9 mm and 26.6 cm. Five (5) of the specimens were preserved in 96% alcohol solution (Hasan & Taman 2019) and transported to the Hydrobiology Laboratory, Universitas Brawijaya, Malang, Indonesia (voucher no. Hb.Pm.I.2019). The remaining eleven (11) specimens were kept as livestock at the Fish Reproduction Laboratory, Universitas Brawijaya, Malang, Indonesia. The 11 living specimens were transported in oxygen-filled polyethylene bags.

Diagnosis

The morphological characters of the specimens are as follows: a large mouth, projecting lower jaw, prominent enlarged canine teeth, a more or less continuous black stripe between the eye and opercular margin, and another stripe between the eye and the lower angle of the opercle, and a row of black blotches along the middle of the side.

The fish can be distinguished from other members of the genus by having the expanded preopercle at the angle. It has silvery or golden-green to purple body colors and black spots on the fins and body. There are also numerous black spots on the anal and caudal fins. The fish has moss green back, purple iridescence sides, and a whitish or yellowish belly. It also has whitish yellowish, or blue iridescence dorsal interspaces, and a black blotch on the caudal-fin base.

All of these characteristics were found in every specimen collected from the Karangkates Reservoir, East Java Province, Indonesia (Fig. 2).

Figure 2 Specimen of *Parachromis managuensis* captured on 8 January 2019 in Karangkates Reservoir, East Java Province

Distribution

The discovery of *P. managuensis* in Karangkates Reservoir is the first record of this species in East Java Province. Other discoveries occurred at reservoirs in West Java and Central Java Provinces. The discovery in Karangkates Reservoir represents a discovery in the eastern part of Java Island, which is around 400 km apart from the Central Java Province (Fig. 3). This record is an important contribution to understanding the dispersal of alien fish species in Indonesia.

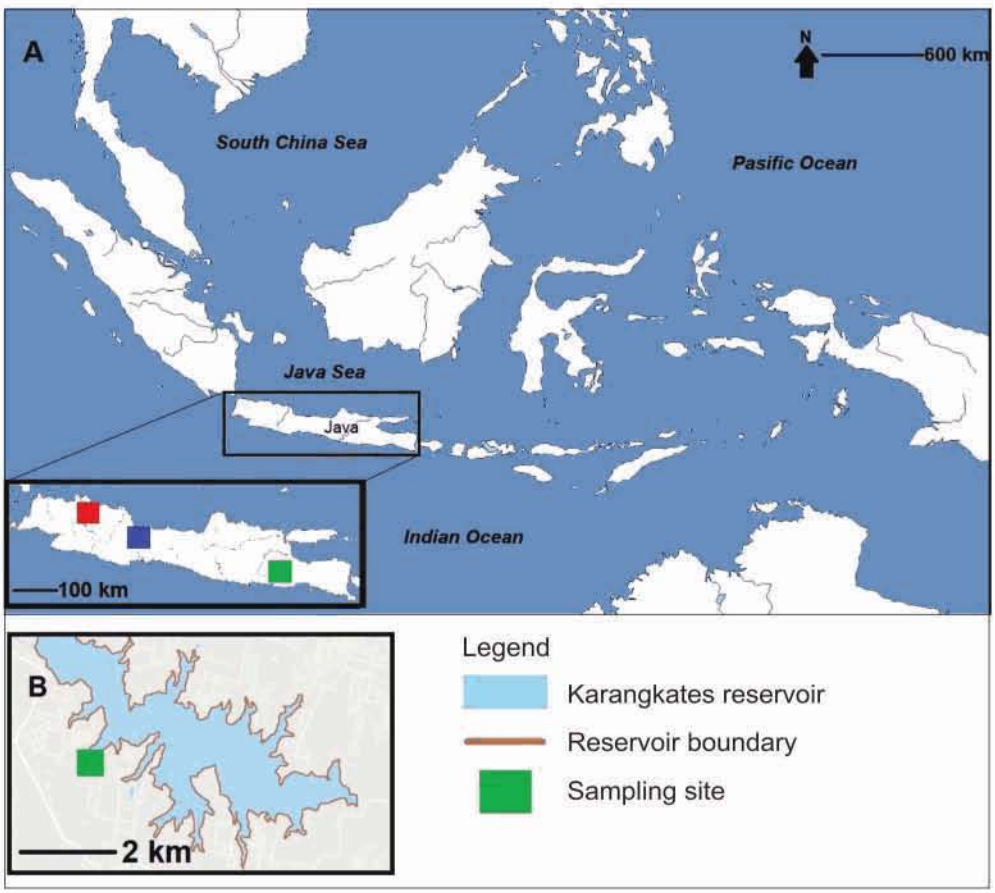

Figure 3 A. Distribution of *Parachromis managuensis* in Java Island
Notes: Red square: West Java Province; Blue square: Central Java Province; Green square: East Java Province).
B. Location of the Karangkates Reservoir in East Java Province
Note: The green square indicates the new record of *P. managuensis*

We speculated that *P. managuensis* were released into Karangkates Reservoir in East Java Province by exotic fish hobbyists without clear purposes. Further investigation is warranted to determine the source of *P. managuensis* in East Java Province because the reservoir has never been used for any exotic fish culture industry. Control and prevention of further introductions are needed to prevent alien fish from disturbing the freshwaters ecosystem (Hasan *et al.* 2020; Wijayanti *et al.* 2021; Hasan *et al.* 2021).

CONCLUSION

Parachromis managuensis is a non-native fish of Indonesia. This fish species has been found in freshwater in West Java and Central Java Provinces and also in the Karangkates Reservoir in East Java Province. The existence of *P. managuensis* in East Java is considered a new finding and added data on the alien fish species distribution in Indonesia.

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REFERENCES

Agasen EV, Clemente JP, Rosana MR, Kawit NS. 2006. Biological investigation of Jaguar Guapote *Parachromis managuensis* (Günther, 1867) in Taal Lake, Philippines. J Environ Sci Manag 9(2):20-30.

Barros LC, Santos U, Zanuncio JC, Dergam JA. 2012. *Plagioscion squamosissimus* (Sciaenidae) and *Parachromis managuensis* (Cichlidae): A threat to native fishes of the Doce River in Minas Gerais, Brazil. PLoS ONE 7(6): e39138. https://doi:10.1371/journal.pone.0039138.

Bussing WA. 1998. Peces de Las Aguas Continentales de Costa Rica (Freshwater Fishes of Costa Rica). 2nd edition. Editorial de la Universidad de Costa Rica, San José.

Conkel D. 1993. Cichlids of North and Central Americas. New York (US): TFH Publications. 191 p.

Dahruddin H, Hutama A, Busson F, Sauri S, Hanner R, Keith P, …, Hubert N. 2016. Revisiting the ichthyo diversity of Java and Bali through DNA barcodes: Taxonomic coverage, identification accuracy, cryptic diversity and identification of exotic species. Mol Ecol Resour 17(2):288-99. https://doi:10.1111/1755-0998.12528.

Hasan V, Widodo, MS, Islamy RA, Pebriani DAA. 2020. New records of alligator gar, *Atractosteus spatula* (Actinopterygii: Lepisosteiformes: Lepisosteidae) from Bali and Java, Indonesia. Acta Ichthyologica et Piscatoria 50(2): 233-236. https://doi:10.3750/AIEP/02954.

Hasan V, Tamam MB. 2019. First record of the invasive Nile Tilapia, *Oreochromis niloticus* (Linnaeus, 1758) (Perciformes, Cichlidae), on Bawean Island, Indonesia. Check List 15 (1): 225-227. https://doi.org/10.15560/15.1.225.

Wijayanti A, Hasan V, Tamam MB. 2021. Range expansion of *Oreochromis niloticus* (Linnaeys, 1758) (Perciformes, Chichlidae) in Java Sea and first record for Masalembo Island. IOP Conference Series Earth and Environmental Science 718(1): 012096.

Hasan V, Valen FS, Islamy RA, Widodo MS, Saptadjaja AM, Islam I. 2021. Short Communication: Presence of the vulnerable freshwater goby *Sicyopus auxilimentus* (Gobiidae, Sicydiinae) on Sangihe Island, Indonesia. Biodiversitas 22: 571-9 https://doi.org/10.13057/biodiv/d220208.

Hedianto DA, Purnomo K, Warsa A. 2013. Interactions of food resources utilization by fish communities in Penjalin Reservoir, Central Java. Bawal 5(1): 33-40.

Fuller PL, Nico LG, Willians JD. 1999. Nonindigenous fish introduced into inland waters of the United States. Bethesda (US): American Fisheries Society.

Gestring KB, Shafland PL. 1997. Status and selected life history attributes of the exotic Jaguar Guapote (*Cichlasoma managuense*) in Florida. Florida Scientist 60(3):137-42.

Kwik JTB, Kho ZY, Quek BS, Tan HH, Teo DCJ. 2013. Urban stormwater ponds in Singapore: Potential pathways for spread of alien freshwater fishes. BioInvasions Rec 2(3):239-45. http://dx.doi.org/10.3391/bir.2013.2.3.11.

Kullander SO, Hartel KE. 1997. The systematic status of cichlid genera described by Louis Agassiz in 1859: *Amphilophus, Baiodon, Hypsophrys* and *Parachromis* (Teleostei: Cichlidae). Ichthyol Explor Freshw 7:193-202.

Magalhães ALB, Jacobi CM. 2013. Invasion risks posed by ornamental freshwater fish trade to southeastern Brazilian rivers. Neotrop Ichthyol 1(3):433-41. https://doi.org/10.1590/S1679-62252013005000003.

Page LM, Burr BM. 1991. A field guide to freshwater fishes of North America and North of Mexico. Boston (US): Houghton Mifflin Company. 432 p.

Rosana MR, Agasen EV, Villanueva LS, Clemente Jr JP, Kawit NS, de la Vega JT. 2006. Status and economic impact of *Parachromis Maraguensis* in Taal Lake, Philippines. J Environ Sci Manag 9(2):1-19.

Sampaio WMS, Belei F, Giongo P, Dergam JA, Orsi ML. 2017. *Heterotilapia buttikoferi* (Hubrecht, 1881) (Perciformes: Cichlidae), an introduced exotic fish in the upper Paraná river basin. Check List 13(4): 245-250. https://doi.org/10.15560/13.4.245.

Stein JA, Shultz AD, Cooke SJ, Danylchuk AJ, Hayward K, Suski CD. 2012. The influence of hook size, type, and location on hook retention and survival of angled bonefish (*Albula vulpes*). Fish Res 113:147-52.

Yamamoto MN, Annete WT. 2000. Hawai'i's Native and Exotic Freshwater Animals. Honolulu (US): Mutual Publishing.

HEAVY METALS CONTAMINATION LEVEL AND WATER QUALITY PARAMETER CONDITIONS IN JATILUHUR RESERVOIR, WEST JAVA, INDONESIA

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ABSTRACT

Waste pollution into the Citarum River, the main water source of Jatiluhur Reservoir, was dominated by the manufacturing industry such as textile, chemical, metal and pharmaceutical. In general, the manufacturing industry is the most common contributor to heavy metal waste, which will cause various health problems. Therefore, it is essential to conduct studies on heavy metal contamination and water quality parameters conditions in the Jatiluhur Reservoir. The study aimed to provide a reference regarding the current condition of the heavy metal contamination level in both sediment and water of the Jatiluhur Reservoir, as well as to compare the levels of other water quality parameters against the standard of environmental quality. Heavy metals contents, such as Cu, Zn, Hg, Pb and Cd, were determined using X-Ray Fluorescence (XRF) Spectrometry method (for sediment) and Atomic Absorption Spectrometry (AAS) method (for water). Water quality parameters were analyzed by using methods developed by the Indonesian National Standard (SNI). The data obtained were compared to the Canadian Sediment Quality Guidelines (for heavy metal in sediment) and water quality standards from the Government of the Republic of Indonesia Regulation Number 82 of 2001 (Class 3) (for water quality parameters). Based on this study, Jatiluhur Reservoir is divided into three zones i.e., the inlet area, main inundation area and outlet area. Within the sedimentary layer, the mercury (Hg) was found to be accumulated throughout the Jatiluhur Reservoir area, exceeding the maximum limit, while Cu accumulated in the inlet area, exceeding the minimum limit. The other heavy metals (Zn, Pb and Cd) were found to be exceeding the minimum limit at some locations, but more results were below the minimum limit. The high concentration of heavy metals in the sediment was due to household and/or industrial wastes. Although all heavy metals were not detected in water, the presence of heavy metals in sediments could potentially dissolve into the water by means of upwelling. If this happens, the heavy metals can be excessively contained in water, resulting in a harmful habitat for aquatic biota. Water containing heavy metals will also be harmful for human. In general, water quality parameters in Jatiluhur Reservoir meet the standard for water quality. Only ammonia, however, was higher than the standard for sensitive fish life due to massive aquaculture activities in this reservoir. Considering the conditions of heavy metal contamination levels in both sediment and water, the biota that is most likely to be exposed to heavy metal is benthic organisms, because the organisms live at the bottom of the waters. The priority for further attention and countermeasure in improving the sediment and water quality of the Jatiluhur Reservoir was toward Hg, Cu, and Pb and ammonia.

Keywords: heavy metal, reservoir, sediment, water

INTRODUCTION

Jatiluhur Reservoir was the largest reservoir in Indonesia having an area of 8,300 ha. Jatiluhur Reservoir was built in 1957. The reservoir is the first multipurpose reservoir in Indonesia with potential available water of 12.9 billion m^3/year. The Jatiluhur Reservoir was built by blocking the Citarum River. The river passes through various anthropogenic activities and many cities in the West Java Province. Among functions of the Jatiluhur Reservoir are as hydropower, as a source of irrigation for 242,000 ha of surrounding rice fields, as a source of drinking water, as a place for inland fisheries (fish farming in floating net cages (KJA)), as flood control, and as tourism and water sports facilities. The types of fish commonly being

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cultivated in the floating net cages (KJA) in the Jatiluhur Reservoir include common carp, nile tilapia, red devils and catfish (*Pangasius* spp.) (Suprian & Salami 2011).

In the past few years, fishes in the Jatiluhur Reservoir seem to have been contaminated and are not suitable for consumption (Republika.co.id 2009). Likewise, in 2018, a report from Anggoro (2018) stated that there was an appeal for not consuming any kinds of fish from this reservoir. The contamination is allegedly due to the influence of various pollutions from the Citarum River as the main water source of the Jatiluhur Reservoir. The contamination may have also occurred due to the wastes of fish feed from the many KJAs in the reservoir. Industrial activities are the most common contributor of heavy metal waste because heavy metals are used in industrial activities as raw materials, additives and catalysts (Hutagalung *et al.* 1997 in Vigers *et al.* 1996). Heavy metal contamination in waters causes various health problems for aquatic biota and humans, such as problems in the nervous system, respiratory system, liver function, kidney function and growth of bones (Sanusi 1985). Heavy metals are categorized as harmful pollutants because it cannot be destroyed (non-degradable) by living organisms, so they will settle at the bottom of the waters and accumulate (Rochyatun & Rozak 2007).

Heavy metals can bond with organic compounds to form complex compounds that eventually settle at the bottom of the water (accumulate in sediments) (Marchand *et al.* 2006). On the other hand, sediments were an inseparable part of aquatic ecosystems that can provide habitat, feeding grounds, spawning grounds, and nurseries for various aquatic organisms. Contaminated sediments can reduce or eliminate the aquatic organisms that have important values for ecology, commercial or recreational uses (US EPA 2001). Fatoki and Mathabatha (2001) stated that sediment has a function as a metal container which can release the metal into the water through natural and anthropogenic processes. Heavy metals deposited in sediments can cause changes in water quality and transfer toxic chemicals to aquatic organisms (Permanawati *et al.* 2013).

Physical and chemical water quality parameters such as dissolved oxygen (DO), pH, total organic content, temperature and dissolved ion affect the life of aquatic organisms (Effendi 2003). Water quality parameters is influenced by its catchment area which is related to human activities (Wiwoho 2005; Asrini 2017).

A study conducted by Garno (2002) showed that the Jatiluhur Reservoir was hypertrophic (very nutrient-rich) and phytoplankton bloom can occur any time. The hypertrophic condition was largely influenced by the aquaculture activities in this reservoir and the influx of organic matters from settlements. Poor water quality conditions can disrupt aquatic life in the reservoir and reduce the diversity of aquatic biota. A study in Cirata Reservoir carried out by Komarawidjaja *et al.* (2005) showed that there was a growth disturbance in common carp which was thought to be closely related to water quality, especially with the high concentration of chlorophyll-*a* and total N in the waters. Poor water quality of the Jatiluhur Reservoir will also have an adverse impact on humans due to the function of the reservoir as a source of drinking water and as tourism and water sports facilities.

Based on the issues above, the study of heavy metal contamination and water quality parameter conditions in the Jatiluhur Reservoir should be conducted due to the serious polluted condition in the reservoir. This study aimed to obtain data on current condition of the heavy metal contamination level that has occurred in both sediment and water, as well as to compare the levels of other water quality parameters against the standard of environment quality.

MATERIALS AND METHODS

Location and Time

The research was conducted in Jatiluhur Reservoir in February 2019. Water and sediment samples were taken from 6 (six) locations in the reservoir, consisting of 3 (three) zones i.e., the inlet area, main inundation area, and outlet area (Fig. 1). The six locations were: 1. Citarum River inlet; 2) Jamaras; 3) Cilalawi River inlet; 4) KJA zone 5; 5) Pasir Kole and 6) DAM (outlet).

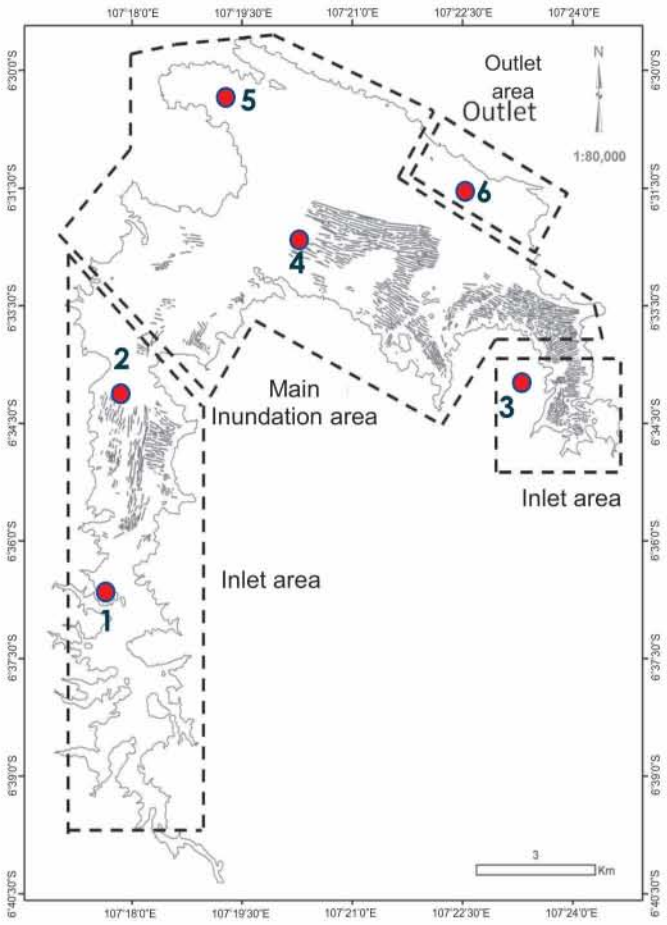

Figure 1 Map of sampling locations in Jatiluhur Reservoir

Methods

The water sample was taken using Van Dorn Water Sampler, then analyzed in the Global QA Laboratory using the methods from the Indonesian National Standard (SNI). Sediment sampling was conducted by using an Ekman Grab. The sediment samples obtained were then analyzed in the Central Nuclear Material Technology Laboratory, BATAN using the X-Ray Fluorescence Spectrometry (XRF) method to find out the heavy metals (Cu, Zn, Hg, Pb and Cd) contents. The XRF Spectrometry method is an application of radioisotopes used as an analytical method for detecting heavy metal content, especially in solid substances such as sediment. This method is still not commonly used in aquatic ecological studies that mostly use the AAS method. Methods used for analyzing the water and sediment samples are presented in Table 1.

Data Analyses

Data of heavy metal contents (in water and sediment) and water quality parameters obtained from this study were compared to the quality standard. The sediment parameters (heavy metal) were compared to the Canadian Sediment Quality Guidelines for the Protection of Aquatic Life on Freshwater set out by the Canadian Council of the Ministry of Environment (CCME). Reference to heavy metal quality standards according to CCME standard was presented in Table 2. Data on water quality parameters were compared to water quality standards from the Government Regulation of the Republic of Indonesia Number 82 of 2001 (Class 3, for the cultivation of freshwater fish, animal husbandry, water to irrigate crops, and or other purposes that require the same water as these uses).

Table 1 Parameters and methods used for analyzing water and sediment samples

Parameter	Unit	Method
A. Water		
Physical Properties:		
Temperature[a]	°C	5.4/IK/GQA/WQ/002
Total Dissolved Solid, TDS[a]	mg/L	SNI 06-6989.27-2005
Total Suspended Solid, TSS[a]	mg/L	SNI 06-6989.3-2004
Chemical Properties:		
pH[a]	-	SNI 06-6989.11-2004
Biological Oxygen Demand, BOD_5[a]	mg/L	SNI 6989.72:2009
Chemical Oxygen Demand, COD[a]	mg/L	SNI 6989.2:2009
Dissolved Oxygen, DO[a]	mg/L	SNI 06-6989.14-2004
Total Phosphate as P[a]	mg/L	5.4-IK-GQA-WQ-062
Nitrogen, Nitrate as N (NO_3-N)[a]	mg/L	5.4-IK-GQA-WQ-043
Ammonia, NH_3-N[a]	mg/L	SNI 06-6989.30-2005
Copper, Cu[a]	mg/L	SNI 6989.6:2009
Zinc, Zn[a]	mg/L	SNI 6989.7-2009
Mercury, Hg[a]	mg/L	SNI 6989.78:2009
Lead, Pb[a]	mg/L	SNI 6989.46:2009
Cadmium, Cd[a]	mg/L	SNI 06-6989.38-2005
B. Sediment		
Copper, Cu	µg/g	XRF Spectrometry
Zinc, Zn	µg/g	XRF Spectrometry
Mercury, Hg	µg/g	XRF Spectrometry
Lead, Pb	µg/g	XRF Spectrometry
Cadmium, Cd	µg/g	XRF Spectrometry

Note: [a] = Accredited by the National Accreditation Committee (KAN).

Table 1 Quality standards for heavy metals in sediments (for freshwater) based on CCME (2001)

Heavy metal	Concentration (mg/kg dry wt.)	
	ISQG[a]	PEL[b]
Cu	35.7	197.0
Zn	123.0	315.0
Hg	0.170	0.486
Pb	35.0	91.3
Cd	0.6	3.5

Notes: a = ISQG (Interim Sediment Quality Guidelines): the value of the minimum limit, i.e., the limit of metal concentrations that has the low possibility to cause a negative biological effect; b = PEL (Probable Effect Level): the value of the maximum limit, i.e., the limit of metal concentrations having a greater possibility to cause a negative biological effect.

RESULTS AND DISCUSSION

Heavy Metal Contaminations

Our study showed that all heavy metals (Cu, Zn, Hg, Pb, and Cd) accumulated in the sediment layer of the Jatiluhur Reservoir and had exceeded the minimum limit (ISQG). Mercury (Hg) concentration exceeded the maximum limit (PEL) throughout the Jatiluhur Reservoir area. Copper (Cu) tended to accumulate in the inlet area, with concentration exceeding the minimum limit. The concentration of Zinc (Zn) and lead (Pb) exceeded the minimum limit at some locations (such as sampling point 5), however, the concentration of Cadmium was below the detection limit of the measuring tools (Table 3). Cadmium (Cd) seems to be relatively the safest metal found in the sediment samples compared to the other heavy metals.

Table 3 Results of laboratory analysis for heavy metal in sediment samples

Station	Test	Element (mg/kg)				
		Cu	Zn	Hg	Pb	Cd
1 Citarum River inlet	#1	39.78	105.16	2.1	29.4	<0.3
	#2	40.34	96.32	2.5	29.7	<0.3
	#3	35.71	93.51	1.8	28.6	<0.3
2 Jamaras	#1	36.91	113.59	1	26.8	<0.3
	#2	38.42	106.6	<0.7	26.8	<0.3
	#3	41.46	133.2	2.5	28.8	<0.3
3 Cilalawi River inlet	#1	35.79	129.82	<0.7	34.8	<0.3
	#2	84.12	103.31	2.4	31.9	<0.3
	#3	73.73	92.06	3.2	30.7	<0.8
4 KJA zone 5	#1	22.3	114.48	1.1	30.2	<0.8
	#2	12.94	104.27	2.5	25.9	<0.3
	#3	19.01	113.03	3.8	34	<0.3
5 Pasir Kole	#1	18.69	139.94	2.2	40	<0.3
	#2	23.57	143.16	1.5	36.3	<0.3
	#3	26.28	147.98	3.2	40.1	<0.3
6 DAM (outlet)	#1	20.13	89.41	<0.7	35.3	<0.3
	#2	74.13	103.39	3.6	32.5	<0.8
	#3	27	97.69	<0.7	31	<0.3
Quality Standard[a]		35.7	123	0.170	35.0	0.6
		197.0	315	0.486	91.3	3.5

Notes: a = Based on the Canadian Sediment Quality Guidelines for the Protection of Aquatic Life on Freshwater (CCME 2001).

exceed the ISQG (minimum limit)

exceed the PEL (maximum limit)

Mercury (Hg) was among the most toxic metals for aquatic biota. Mercury can be released from anthropogenic activities, such as from the antifouling paint for the hull of ships, slimicides used in the lumber and paper industries, pesticides and seed dressings in agriculture and pharmaceuticals (Garcia-Rico *et al.* 2006). Mercury has many benefits, but it is very toxic and has a high level of bioaccumulation capabilities (Garcia-Rico *et al.* 2006; Palar 2012). Mercury concentration exceeding the safe limit endangers the life of aquatic biota, either directly or indirectly (Palar 2012). Therefore, the high concentration of mercury in the Jatiluhur Reservoir should be handled immediately, especially when there is an upwelling process in the reservoir.

Our study showed that the concentration of Cu in sediments was much higher and exceeded the minimum limit in inlet areas, whereas the concentration of Zn and Pb was high in the main inundation areas. The spatial difference of the heavy metals' concentrations might be due to the heavy influx from the river into the reservoir, which was already contaminated by various anthropogenic activities. Cu is commonly used in insecticides, fungicides, brass alloy materials for household appliances, machine parts, as well as in water purification or as a food additive (Palar 2012). Household wastes in the form of metabolic waste and corrosion of pipes in residential areas usually also contain Cu (Connell & Miller 2006). The main inundation area has the longest water retention time compared to the other areas and is also much affected by the KJA aquaculture activities (marked with black lines in Fig. 1). Household wastes, water flow from urban areas and phosphate utilization (PO_4), contributed significantly to the entry of Pb metal into the waters (Connell & Miller 2006; Harteman 2011). Sources of Zn, Cu and Pb are chemical fertilizers, household wastes such as corrosion of pipes and detergent, as well as industrial wastes such as battery materials, cosmetics, plastics, rubber, soap, paint and ink, television tubes and fluorescent lamps, deodorants and chemicals for wood preservation (Connell & Miller 2006).

Table 4 Results of laboratory analysis results for heavy metal in water samples

Station	Element (mg/L)				
	Cu	Zn	Hg	Pb	Cd
1. Citarum River inlet	< 0.006	< 0.004	< 0.00009	< 0.0002	< 0.00004
2. Jamaras	< 0.006	< 0.004	< 0.00009	< 0.0002	< 0.00004
3. Cilalawi River inlet	< 0.006	< 0.004	< 0.00009	< 0.0002	< 0.00004
4. KJA zone 5	< 0.006	< 0.004	< 0.00009	< 0.0002	< 0.00004
5. Pasir Kole	< 0.006	< 0.004	< 0.00009	< 0.0002	< 0.00004
6. DAM (outlet)	< 0.006	< 0.004	< 0.00009	< 0.0002	< 0.00004
Quality Standard[a]	0.020	0.050	0.00200	0.0300	0.01000

Notes: a = Based on Government Regulation of the Republic of Indonesia Number 82 of 2001 (Class 3).

The concentrations of heavy metals in water in this study were very low, below the detection limit of the tool (Table 4), which indicated that the concentrations of heavy metals (Cu, Zn, Hg, Pb, and Cd) in the water of the Jatiluhur Reservoir were still within the safe limits. This fact was in agreement with a study in Jatiluhur Reservoir in 2011 conducted by Suprian and Salami (2011), especially for mercury concentration which was below the quality standard of the Government of the Republic of Indonesia Number 82 of 2001 (Class 3). Despite the results that all heavy metals in the water samples were below the safe limit, however, heavy metals in sediments could potentially dissolve into the water. Suprian and Salami (2011) stated that although the value of mercury in water is below the safe limit, however, the existence of mercury has to be monitored because it is difficult to eliminate mercury from the water. According to Fatoki and Mathabatha (2001) and Permanawati *et al.* 2013, sediment can release heavy metal into the water through natural and anthropogenic processes that cause changes in water quality and transfer of toxic chemicals to aquatic organisms. In reservoirs, the most extreme natural process that allows this to happen is upwelling.

Palar (2012) stated that the 0.01 ppm concentration of Cu in water is deadly for phytoplankton, Pb concentration of 2.75-49 ppm is deadly for crustaceans, while Pb concentration of 188 ppm is deadly for fish, and Cd concentration of 0.0028-4.6 ppm is deadly for Oligochaeta. Mercury concentration of ≥ 0.16 ppm is able to reduce the survival and growth rates of fish caused by the increase of stress and organ damage, whereas mercury concentration of ≥ 3 ppm causes mass mortality in common carp (Nirmala *et al.* 2012; Tyas *et al.* 2013). Lethal toxicity test by Prayogo *et al.* (2016) showed that 96 hours mercury exposure with concentration of 0.396 ppm in water had a 50% lethal effect on nilem carp (*Osteochilus hasselti*). If the heavy metals content in the sediment are released into the water, then the concentration of heavy metals can be excessive in the water, causing adverse impact to humans and aquatic biota.

Considering the conditions of heavy metal contamination levels in both sediment and water, the biota that is most likely to be exposed to heavy metal is benthic organisms, because benthic organisms live at the bottom of the waters (Reynolds 2012). Heavy metals can cause a decrease in species richness of benthic organisms and a change in species composition of benthic macroinvertebrate communities (Qu *et al.* 2010). Cu concentrations of 70-90 mg/kg, Zn concentrations of ±350 mg/kg and Pb concentrations of 30-40 mg/kg caused the decrease of Polychaeta and Molluscs biodiversity at the bottom of Jakarta Bay, especially in industrial areas (Takarina & Adiwibowo 2011). The same occurrences might happen in the Jatiluhur Reservoir, especially for Cu and Pb which concentrations have reached high level of contamination. Qu *et al.* (2010) stated that even though contamination by heavy metals is low in the sampling area, the adverse impact on benthic organisms are significant, suggesting that the chronic effects of long-term exposure to heavy metals in aquatic communities could be serious. Thus, it is essential to focus our attention and efforts to handle contamination of Hg, Cu, and Pb in the Jatiluhur Reservoir.

Water Quality Parameter Conditions

In general, water quality parameters in our study met the quality standards (QS) based on the Government of the Republic of Indonesia Regulation No. 82 of 2001 (Class 3). Based on direct measurements on-site for all sampling locations, the temperature ranged from 26.5 °C up to 27.1 °C (QS ± 3), Dissolved Oxygen (DO) concentrations ranged from 4.2 mg/L up to 4.5 mg/L (QS min. 3 mg L), and the water pH ranged from 6.37 up to 7.17 (QS 6-9). Results of the laboratory analysis on the other parameters of water quality are presented in Figure 2.

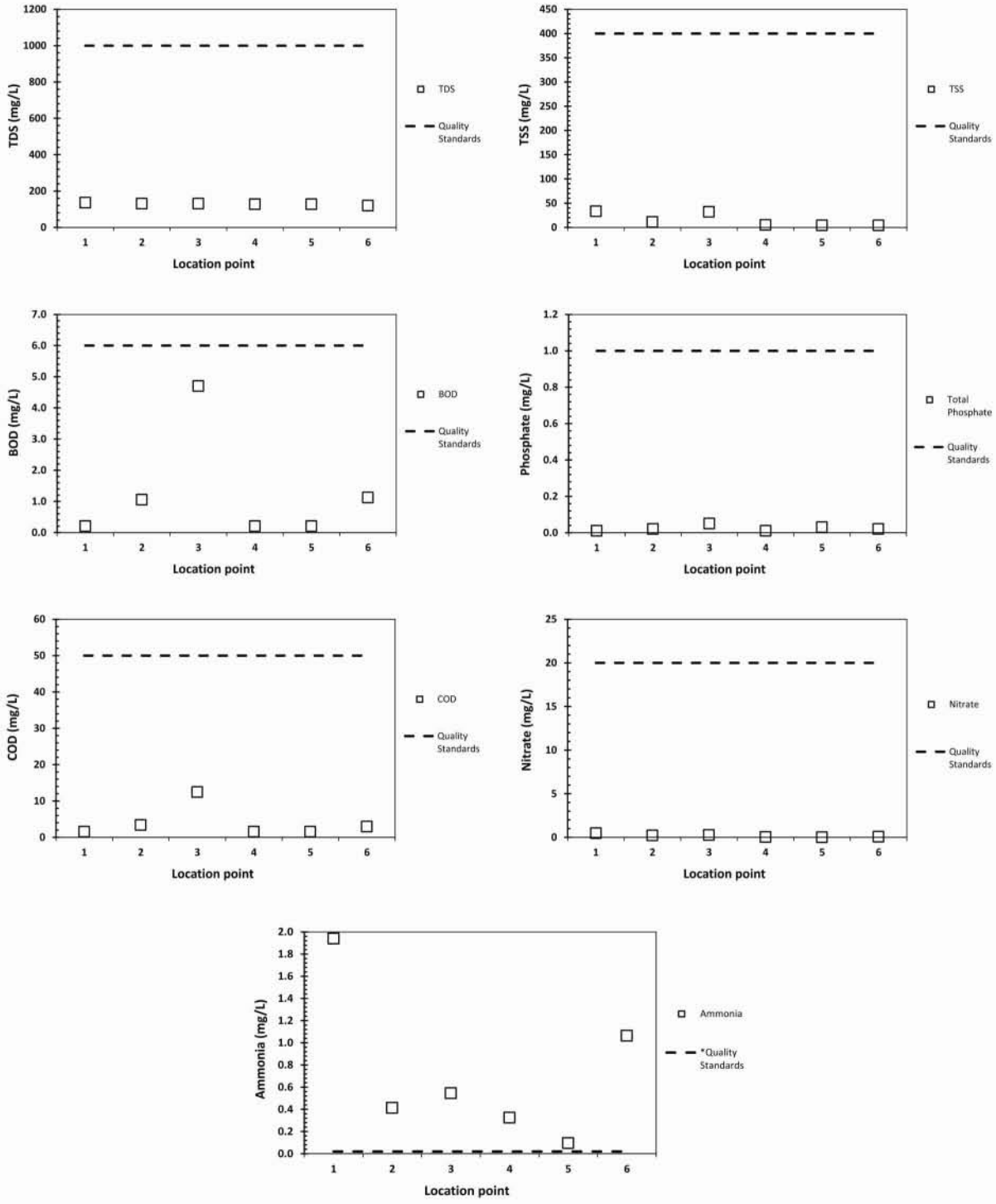

Figure 2 Values of several water quality parameters measured in the Jatiluhur Reservoir
Note: Quality Standards based on the Government of the Republic of Indonesia Regulation No. 82 of 2001 (Class 3), except for ammonia which was not available.

Results of our study showed that TDS concentrations ranged from 120 mg/L up to 136 mg/L (QS 1,000 mg/L), TSS concentrations ranged from 4 mg/L up to 33 mg/L (QS 400 mg/L), BOD concentrations ranged from 0.2 mg/L up to 4.7 mg/L (QS 6 mg/L), COD concentrations ranged from 2 mg/L up to 12 mg/L (QS 50 mg/L), total phosphate concentrations ranged from 0.01 mg/L up to 0.10 mg/L (QS 1 mg/L), nitrate concentrations ranged from 0.01 mg/L up to 0.47 mg/L (QS 20 mg/L) and ammonia concentrations ranged from 0.095 mg/L up to 1.940 mg/L (QS 0.02 mg/L).

Of the ten observed water quality parameters (not including heavy metals concentrations), only ammonia that did not meet the quality standards of water quality parameters. Ammonia is a nitrogen compound that changes to NH_4 ions at low pH conditions. Ammonia can also come from domestic and industrial wastes (Marganof 2007).

The high content of ammonia in the reservoir was presumably due to the fish feed and fish fecal wastes as a result of aquaculture activities in the reservoir. Fish emit 80-90% ammonia (N-inorganic) through the osmoregulation process, while feces and urine account for 10-20% of total nitrogen (Rakocy *et al.* 1992 in Sumoharjo 2010). Ammonia in the reservoir can come from organic and inorganic nitrogen sources found in soil and water or from the decomposition of organic matters by microbes and fungi. Ammonia also comes from the denitrification process during the decomposition of wastes by microbes under anaerobic conditions (Effendi 2003). Commonly, the concentration of Ammonia in the pond should not exceed 0.05 mg/L. According to SNI 6139:2009 (National Standardization Agency 2009), the ammonia value resulting from the nile tilapia aquaculture activity in calm water ponds should not exceed 0.02 mg/L. Ammonia concentrations of 0.02-0.07 mg/L have been shown to inhibit growth and cause tissue damage in several fish species. The toxicity threshold value for ammonia is highly dependent on the type of species, size, fine solids, surface-active compounds, metals and nitrates (Colt 2006).

Based on Anas *et al.* (2017), the water quality status of the Jatiluhur Reservoir is classified as moderate. The moderate status was resulted from the STORET calculation, stated in the Decree of the Minister of Environment No. 115 of 2003) which was caused by high concentrations of BOD, COD and ammonia. The main contributor to the high concentrations of organic matter in the Jatiluhur Reservoir presumably are the number of operating KJA in the reservoir. Based on the Regent of Purwakarta Regency Decree No. 6 of 2000, the optimal number of KJA to operate in the Jatiluhur Reservoir is 2,100 plots. Meanwhile, in 2015 there were 18,038 KJA in the reservoir, which exceeded the carrying capacity of the waters (Astuti *et al.* 2016). A recent study conducted by Fitri *et al.* (2016) stated that in 2014 the number of intensive KJA in the Jatiluhur Reservoir was already excessive, amounting to 23,000 cages. According to their study, the optimal number of KJA was 19,401 plots. Moreover, Fitri *et al.* (2016) stated that the difficulties faced by the Jatiluhur Reservoir related to the KJA problem were caused by multi-parties' management having different perspectives leading to inconsistent decision making. Harmonious perspectives and visions of all managing parties are needed to maximize the productivity of the KJA without sacrificing environmental quality.

CONCLUSION

The Jatiluhur Reservoir has experienced the accumulation of Cu, Zn, Pb, and Cd in the sediment layer with exceeding the minimum limit (ISQG) and Hg with exceeding the maximum limit (PEL). Spatially, Hg concentration was high in the entire Jatiluhur Reservoir area, Cu was found to accumulate in the inlet area, whereas Zn and Pb were relatively high in the main inundation areas. Cadmium (Cd) seemed to be relatively the safest metal in sediment compared to the other heavy metals. Heavy metal concentration in the waters of Jatiluhur Reservoir was below the detection limit of the tool. The other water quality parameters also met the standard of water quality. Only ammonia did not meet the quality standards for the life of sensitive fish (such as Nile tilapia). The high concentration of heavy metals in the sediment of the reservoir was due to household

and/or industrial wastes, while the high concentration of ammonia in water was due to fecal materials from the aquaculture activities. Benthic organisms may have been affected by the high concentration of heavy metals in the sediment of the reservoir. Based on the high level of heavy metal concentrations in the Jatiluhur Reservoir, the priority for further attention and countermeasure in the reservoir was toward Hg, Cu, Pb and ammonia. Further studies are recommended to manage the water quality of the Jatiluhur Reservoir.

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REFERENCES

Anas P, Jubaedah L, Sudino D. 2017. Kualitas air dan beban limbah karamba jaring apung di Waduk Jatiluhur Jawa Barat [Water quality and wastewater load of floating net cages in Jatiluhur Reservoir of West Java]. JPPIK 11(1):35-47.

Anggoro B [Internet]. 2018. Warga diimbau tak konsumsi ikan dari aliran Sungai Citarum [Residents are advised not to consume fish from the Citarum River]. [updated 2018 Mar 02; cited 2020 sep 25]. Available at: https://mediaindonesia.com/read/detail/147562-warga-diimbau-tak-konsumsi-ikan-dari-aliran-sungai-citarum.

Asrini NK, Adnyana IWS, Rai IN. 2017. Studi analisis kualitas air di daerah aliran sungai Pakerisan Provinsi Bali [Water quality analysis study in the Pakerisan watershed, Bali Province]. Ecotrophic 11(2):101-07.

Astuti LP, Nurfiarini AN, Sugianti Y, Warsa A, Rahman A, Hendrawan ALS. 2016. Tata kelola perikanan berkelanjutan di Jatiluhur [Sustainable fisheries management in Jatiluhur]. Yogyakarta (ID): Deepublish.

[CCME] Canadian Council of the Ministry of Environment. 2001. Canadian sediment quality guidelines for the protection of aquatic life: summary table. updated from: Canadian environmental quality guidelines. 1999. Winnipeg (CA): Canadian Council of Ministers of the Environment. p. 1-4.

Colt J. 2006. Water quality requirements for reuse systems. Aquaculture Engineering 34:143-56.

Connell DW, Miller GJ. 2006. Kimia dan ekotoksikologi pencemaran. Koestoer Y, translator. Jakarta (ID): UI Press. Translated from: Chemistry and Ecotoxicology of Pollution. p. 346.

Decree of the Regent of Purwakarta Regency No. 6 of 2000 about the utilization of reservoirs for fishery activities. Purwakarta (ID).

Decree of the Minister of Environment No. 115 of 2003 about guidelines for determining water quality status. Jakarta (ID).

Effendi H. 2003. Telaah kualitas air: bagi pengelolaan sumber daya dan lingkungan perairan [Water quality assessment: for the management of aquatic resources and the environment]. Yogyakarta (ID): Kanisius.

Fatoki OS, Mathabatha S. 2001. An assessment of heavy metal pollution in the East London and Port Elizabeth harbours. Water SA 27(2):233-40.

Fitri N, Hidayat A, Gandhi P. 2016. Daya dukung lingkungan dan kelembagaan usaha keramba jaring apung (KJA) di Waduk Jatiluhur [Environmental carrying capacity and institutional floating net cage (KJA) in Jatiluhur Reservoir]. Jurnal Risalah Kebijakan Pertanian dan Lingkungan 3(3):248-61.

Garcia-Rico L, Rodríguez MV, Jara-Marini ME. 2006. Geochemistry of mercury in sediment of oyster areas in Sonora, Mexico. Marine Pollution Bulletin 52(4):453-8.

Government of the Republic of Indonesia Regulation No. 82 of 2001 about water quality management and water pollution control. Jakarta (ID).

Harteman E. 2011. Dampak kandungan logam berat terhadap kemunculan polimorfisme ikan badukang (*Arius maculatus*, Fis & Bian) dan Sembilang (*Plotosus canius*, Web & Bia) di Muara Sungai Kahayan serta Katingan, Kalimantan Tengah [Impact of heavy metal content on the emergence of polymorphisms in badukang fish (*Arius maculatus*, Fis & Bian) and Sembilang fish (*Plotosus canius*, Web & Bia) polymorphisms in the Kahayan and Katingan River Estuary, Central Kalimantan] [Dissertation]. Retrieved from IPB University.

Hutagalung HP, Setiapermana D, Khozanah. 1997. Organochlorine, oil and heavy metals in Siak estuary, Riau, Indonesia. In: Vigers G, Ong KS, McPherson C, Millson N, Watson I and Tang A (eds). ASEAN Marine Environmental management: Quality Criteria and Monitoring for Aquatic Life and Human Health Protection. Proceedings of the ASEAN Canada Technical Conference on Marine Science (24-29 June 1996), Penang, Malaysia. EVS Environmental Consultants, North Vancouver and Department of Fisheries Malaysia. 817 p.

Komarawidjaja W, Sukimin S, Arman E. 2005. Status kualitas air Waduk Cirata dan dampaknya terhadap pertumbuhan ikan budidaya [Water quality status of Cirata Reservoir and its impact on the growth of cultivated fish]. J Tek Ling 6(1):268-73.

Marchand C, Lallier-Verges E, Baltzer F, Alberic P, Cossa D, Baillif P. 2006. Heavy metals distribution in mangrove sediments along the mobile coastline of French Guiana. Marine Chemistry 98(1):1-17.

Marganof. 2007. Model pengendalian pencemaran perairan di Danau Maninjau Sumatera Barat [Water pollution control model in Lake Maninjau, West Sumatera] [Dissertation]. Retrieved from IPB University.

Maslukah L. 2006. Konsentrasi logam berat Pb, Cd, Cu, Zn dan pola sebarannya di Muara Banjir Kanal Barat, Semarang [Concentrations of heavy metals Pb, Cd, Cu, Zn and their distribution patterns in the mouth of the west flood canal, Semarang] [Thesis]. Retrieved from IPB University.

National Standardization Agency. 2009. SNI 6139:2009: Produksi induk ikan nila hitam (*Oreochromis niloticus* Bleeker) kelas induk pokok [SNI 6139:2009: Main production of black tilapia (*Oreochromis niloticus* Bleeker) parent class]. Jakarta (ID): National Standardization Agency.

Nirmala K, Hastuti YP, Yuniar V. 2012. Toksisitas merkuri (Hg) dan tingkat kelangsungan hidup, pertumbuhan, gambaran darah dan kerusakan organ pada ikan nila *Oreochromis niloticus* [Toxicity of mercury (Hg) on survival and growth rate, hemato- and histopathological parameters of *Oreochromis niloticus*]. Jurnal Akuakultur Indonesia 11(1):38-48.

Nugraha WA. 2009. Kandungan logam berat pada air dan sedimen di perairan Socah dan Kwanyar, Kabupaten Bangkalan [Heavy metal content in water and sediment in the waters of Socah and Kwanyar, Bangkalan Regency]. Jurnal Kelautan. 2(2):158-64.

Palar H. 2012. Pencemaran dan toksikologi logam berat, cet. ke-4 [Heavy metal pollution and toxicology, 4th ed.]. Jakarta (ID): Rineka Cipta. p. 1-152.

Permanawati Y, Zuraida R, Ibrahim A. 2013. Kandungan logam berat (Cu, Pb, Zn, Cd, dan Cr) dalam air dan sedimen di perairan Teluk Jakarta [Heavy metal content (Cu, Pb, Zn, Cd, and Cr) in water and sediment in Jakarta Bay Waters]. Jurnal Geologi Kelautan 11(1):9-16.

Pratiwi NTM, Wijaya HK, Adiwilaga EM, Pribadi TA. 2011. Komunitas perifiton serta parameter fisika-kimia di perairan sebagai penentu kualitas air di Hulu Sungai Cisadane [Periphyton communities and aquatic physico-chemical condition as indikator of water quality of Upstream Cisadane River]. Jurnal Lingkungan Tropis 5(1):21-32.

Prayogo NA, Hidayati A, Siregar AS, Yunasfi. 2016. Uji toksisitas letal dan subletal logam berat merkuri (Hg) terhadap ikan nilem (*Osteochilus hasselti*) [Lethal and subletal toxicity test of heavy metal mercury (Hg) against nilem carp fish (*Osteochilus hasselti*)]. Omni-Akuatika 12(1):86-94.

Qu X, Wu N, Tang T, Cai Q, Park YS. 2010. Effects of heavy metals on benthic macroinvertebrate communities in high mountain streams. Ann Limnol Int J Lim 46 (4):291-302.

Republika.co.id [Internet]. 2009. Ikan dari Waduk Jatiluhur diduga tak layak konsumsi [The fish from Jatiluhur Reservoir are thought to be unfit for consumption]. [updated 2009 Oct 17; cited 2019 Oct 1]. Available at: http://www.republika.co.id/berita/breaking-news/nusantara/09/10/17/82917-ikandariwadukjatiluhur-diduga-tak-layak-konsumsi.

Reynolds CS. 2012. Benthic ecology. In: El-Shaarawi AH, Piegorsch WW, editros. Encyclopedia of Environmetrics Second Edition. John Wiley & Sons, Ltd. https://doi.org/ 10.1002/9780470057339.vab010.pub2

Rochyatun E, Rozak A. 2007. Pemantauan kadar logam berat dalam sedimen perairan Teluk Jakarta [Monitoring of heavy metal levels in the sediments of Jakarta Bay waters]. Makara Sains 11(1):28-36.

Sanusi HS. 1985. Akumulasi logam berat Hg dan Cd pada tubuh ikan bandeng (*Chanos chanos* Forsk.) [Accumulation of heavy metals Hg and Cd in milkfish bodies (*Chanos chanos* Forsk.)] [Dissertation]. Retrieved from IPB University.

Sumoharjo. 2010. Penyisihan limbah nitrogen pada pemeliharaan ikan nila (*Oreochromis niloticus*) dalam sistem akuaponik: Konfigurasi desain bioreaktor [Removal of nitrogen waste in nile tilapia (*Oreochromis niloticus*) rearing in aquaponics systems: Bioreactor design configuration] [Theses]. Retrieved from IPB University.

Suprian C, Salami IRS. 2011. Akumulasi merkuri (Hg) pada ikan budidaya keramba jaring apung dan ikan liar di Waduk Jatiluhur [Accumulation of mercury (Hg) in fish on cage aquaculture and wild fish at Jatiluhur Reservoir]. Jurnal Teknik Lingkungan 17(2):68-76.

Takarina ND, Adiwibowo A. 2011. Impact of heavy metals contamination on the biodiversity of marine benthic organisms in Jakarta Bay. Journal of Coastal Development 14(2):168-71.

Tyas NM, Siregar AS, Sulistyo I. 2013. Uji toksisitas letal dan subletal merkuri klorida ($HgCl_2$) terhadap ikan mas (*Cyprinus carpio* L.) [The lethal and sub-lethal toxicity test of mercury chloride ($HgCl_2$) on *Cyprinus carpio* L.]. Omni-Akuatika 12(17):1-10.

[US EPA] U.S. Environmental Protection Agency. 2001. Methods for collection, storage and manipulation of sediments for chemical and toxicological analyses: technical manual. EPA 823-B-01-002. Washington DC (US): U.S. Environmental Protection Agency, Office of Water.

Wiwoho. 2005. Model identifikasi daya tampung beban cemaran sungai dengan QUAL2E [Model identification of river pollution load carrying capacity with QUAL2E] [Thesis]. Retrieved from Universitas Diponegoro.

INHIBITORY EFFECT OF *ULIN* WOOD LIQUID SMOKE AND *GOGO* RICE ENDOPHYTIC FUNGI AGAINST PATHOGEN *Pyricularia oryzae*

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ABSTRACT

Diseases in rice plants (paddy) caused by microorganisms such as *Pyricularia oryzae* lead to a decrease in rice production. Therefore, it is essential to find out biological agents for protecting paddy and plants in general, against plant diseases. Liquid smoke and endophytic fungi have been known as biological agents to enhance the protection of plants against disease. The purpose of this study was to determine the ability of liquid smoke, endophytic fungi and the concentrations combinations to suppress the growth of *P. oryzae*. The results showed that liquid smoke concentrations of 0.17% to 1.75% and endophytic fungi filtrate of 2% to 10% showed significant ability against pathogen *P. oryzae*. However, the combination of liquid smoke and endophytic fungi filtrate at selected concentrations (0.17% liquid smoke combined with 2% endophytic fungi filtrate and 0.34% liquid smoke combined with 2% endophytic fungi filtrate) showed no significant inhibition percentage against *P. oryzae* compared to control. In conclusion, this study showed that the respective applications of liquid smoke and endophytic fungi filtrate inhibit the growth of *P. oryzae*.

Keywords: endophytic fungi, inhibition ability, liquid smoke, *P. oryzae*

INTRODUCTION

Rice plants are the most dominating food crop commodity in Indonesia (Andriani 2008). As many as 95% of Indonesians choose rice as a staple food (Norsalis 2011). One of the rice plants cultivated in Indonesia is Gogo rice, which is grown on moorland (a type of habitat with the characteristic of low growing vegetation on acidic soil) or on dry land (Hairmansis *et al.* 2016).

"Gogo" rice Maninjau variety (*Oryza sativa* L. var. *maninjau*) is one of the Indonesian native varieties of brown rice that comes from the area of Lake Maninjau located in the Sumatera Barat Province of Indonesia. This variety is able to live on the dry land of Central Kalimantan, is resistant to leaf blight disease and able to survive in soil with high iron content (BPPP Jateng 2014).

Among microorganisms attacking Gogo rice plants are pathogenic fungi such as *P. oryzae*, *Rhizoctonia solani*, *Helmithosporium sigoideum* and *Cercospora janseana*. *P. oryzae* is one of the main diseases of rice crops due to its impact on reducing rice productivity (Wang *et al.* 2014; Suganda *et al.* 2016). This pathogen causes blast disease and serious damage to panicles (panicle blast) and leaves (leaf blast) of rice plants, where damage to panicles greatly affects rice productivity (Hayashi *et al.* 2019).

Plant resistance to pathogens can be improved by utilizing the interaction of microorganisms with endophytic microbes.

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Endophytic fungi are a symbiotic between fungi and plants, which has a role to protect plants from pathogens by using the compounds produced by the symbiosis. Symbiosis mutualism of endophytic fungi with plants produces secondary metabolites, such as phytohormones, nutrients and colony formations. Some studies state that secondary metabolites produced by endophytic fungi inhibit pathogenic microbes (Lalngaihawmi *et al.* 2019). Therefore, endophytic fungi are potential biological agents to inhibit pathogenic fungi.

Liquid smoke bioactive materials can also be used to prevent disease caused by microorganisms. Liquid smoke is a mixture of solution and colloidal dispersion of wood smoke vapor in water obtained from the process of wood pyrolysis or made from a mixture of pure compounds (Darmadji 2002; Soldera *et al.* 2008; Lee *et al.* 2011). Liquid smoke has good antimicrobial properties because it can inhibit the growth of microbes.

This study used liquid smoke from "Ulin wood" (*Eusideroxylon zwageri* Teijsm. & Binn). Ulin wood has density characteristics and a tight structure of hardwood, containing complex constituent compounds. Several studies reported that one type of hardwood, "alaban" wood (*Vitex pubescens* VAHL.), contains phenol, carboxylic acid and carbonyl that are antimicrobial (Oramahi & Yoshimura 2013). Based on literature studies, "Ulin" wood liquid smoke may have the ability to inhibit *P. oryzae*.

Both endophytic fungi and liquid smoke of "Ulin" wood have the ability to inhibit pathogenic microorganisms. In this study, both were tested both independently and by combining the two. As an initial effort to find one of the solutions to overcome diseases that often occur in rice plants "gogo" Maninjau varieties. This study was aimed to determine the ability of "Ulin" wood liquid smoke, endophytic fungi and their combinations to suppress the growth of *P. oryzae*.

MATERIALS AND METHODS

Sampling

Samples of "Gogo" rice (*O. sativa* L var. *maninjau*) were taken from the same stretch of land in Jaar Village, East Dusun Subdistrict, East Barito District, Central Kalimantan Province. 2°06'53.9" North-South Latitudes 115°15'22.1" West-East Longitudes. Samples consisting of all parts of the Gogo rice plants were taken along with the soil around the roots of the Gogo rice plants, by using a machete. The samples were stored in polybags and transported to the Microbiology Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Lambung Mangkurat.

Isolation, Purification and Identification of Endophytic Fungi

Endophytic fungi were isolated from the roots of the Gogo rice plants, by using surface sterilization method. The roots were washed using running water, then cut to a size of 10 cm. The root cuts were surface-sterilized by using 0.05% bleach for 60 sec and rinsed twice using sterile distilled water. Each part of the root tip was then slightly cut to totally drain the root tip. The root tip was then planted in a sterile Potato Dextrose Agar (PDA) medium and incubated at a temperature of 28 °C for 3-5 days and observed daily. The grown fungi were then purified (Manurung *et al.* 2014; Nurzannah *et al.* 2014). Fungi identification was carried out by using morphological observations, consisting of macroscopic and microscopic observations with reference to the identification book titled "Illustrated Genera of Imperfect Fungi 4th edition" (Barnett & Hunter 1998).

Screening of Endophytic Fungi

Pathogenicity and antagonism tests were used for the screening of endophytic fungi. Pathogenicity test were carried out by using rice seed refering to Waruwu *et al.* (2016). Prior to being used in the pathogenicity test, the surface of the rice seeds (20 grains) were sterilized by soaking the rice seeds in 70% ethanol for 30 sec, followed by soaking in 1% NaOCl for 60 sec. Subsequently, the rice seeds were flushed 3 times in sterile distilled water. After that, the rice seeds were inoculated in PDA medium that had been previously overgrown by 7-day pure isolates of endophytic fungi and then incubated for 2 weeks at room temperature (27-29 °C). Observation on the growing rice sprouts was carried out at the end of incubation. Isolates of endophytic fungi that did not interfere with rice

germination were used for further testing. Seed germination rate was calculated using the formula (Talukdar 2011):

$$Germination\ (\%) = \frac{Number\ of\ germinated\ seed}{total\ number\ seed} \times 100\%$$

A confirmation test for endophytic fungi infection in the roots of rice plants was conducted for germination by using a method of Luqman *et al.* (2015) that has been modified. Prior to being used in the test, the roots of rice plants were washed thoroughly in running water, drained, then soaked in 5.25% NaClO solution for 5 min, then rinsed using distilled water. Subsequently, the roots were soaked in 1% KOH solution for 30 min and then rinsed using distilled water. After that, the roots were then pre-soaked in 1% H_2O_2 solution for 5 min. The coloring stage was started by soaking the roots in 0.5% vinegar solution, followed by being soaked in ink with a ratio of 1:5 for 30 min. Then, the roots were rinsed with distilled water. Finally, the roots of the rice plants were placed on an object glass and covered by a cover glass, and then observed under a microscope with 40x and 100x magnifications.

The antagonism test of endophytic fungi isolates against *P. oryzae* was conducted by using dual culture method (Tomah *et al.* 2020), which put isolates of pathogenic fungi and endophytic fungi on PDA medium in a Petri dish that has been divided into two quadrants. Each isolate was placed at a distance of 3 cm from the edge of the Petri dish and incubated at a temperature of 28 °C for 5-7 days. After incubation, the inhibition percentage of the pathogens was measured using the formula developed by Rabha *et al.* (2014):

$$Inhibition\ (\%) = \frac{diameter\ of\ pathogen\ control\ colony}{diameter\ of\ pathogen\ treatment\ colony} \times 100\%$$

Filtrate Harvesting of Endophytic Fungi

Filtrate production methods used in this study were modified based on Elita *et al.* (2013) and Malinda *et al.* (2015). Endophytic fungi with the highest inhibitory ability obtained in previous tests were inoculated on PDA slant media then incubated for 7 days at 28 °C. After being incubated, the filtrate was harvested by adding 9 mL of sterile distilled water. The fungi surface was then gently wiped with a fine brush. Subsequently, the water suspension and fungi were transferred to a new test tube, then centrifuged at 3,500 rpm for 20 min. Finally, the supernatant was filtered by using a syringe filter with a pore size of 0.45 µm. The filtrate was then used in testing the inhibitory activity of endophytic fungi against pathogens.

Inhibition Test of Liquid Smoke-Endophytic Fungi to *P. oryzae*

Inhibition test of liquid smoke was conducted at various liquid smoke concentrations, i.e., 0.085%, 0.17%, 0.34%, 0.68%, 1.36% and 1.75%, based on a method of Malinda *et al.* (2015) that have been modified. Liquid smoke was obtained from condensation during the production process of "Ulin" wood charcoal by Talasiana Charcoal Production Group, located at Tanah Laut Regency. The liquid smoke was mixed into the Potato Dextrose Agar (PDA) medium. The pathogen isolates were then grown on the mixture of liquid smoke and PDA for 5-7 days with daily observation. The inhibition percentage was calculated by the formula developed by Rabha *et al.* (2014):

$$Inhibition\ (\%) = \frac{diameter\ of\ pathogen\ control\ colony}{diameter\ of\ pathogen\ treatment\ colony} \times 100\%$$

The inhibition test for endophytic fungi was carried out by using the serial dilution method. Endophytic filtrate with concentrations of 2, 4, 6, 8, and 10% was mixed with PDA medium to be used for growing pathogen, and then incubated at 28 °C for 7 days and observed daily. The inhibition percentage was calculated with the same formula as the one used for calculating the inhibition percentage for the liquid smoke.

Liquid smoke and the endophytic filtrate (Ketoconazole) with various concentrations were then combined to be tested for their inhibitory ability against pathogens. Testing methods and measurements of inhibition percentage were conducted by using the same method as for the previous tests.

RESULTS AND DISCUSSION

Endophytic Fungi of *O. sativa* L var. *maninjau* Root

Endophytic fungi obtained from the roots of "Gogo" rice var *maninjau* were coded AP.2,

AP.3, AP.4, AP.7, AP.8, and AP.9. Microscopic observation of the endophytic fungi isolates showed that there are morphological differences. Based on Barnet & Hunter (1998), the six isolates found refers to several species. AP2 is *Curvularia* sp., AP3 and AP7 are *Penicillium* sp., AP 8 is *Geotrichum* sp., AP9 is *Aspergillus* sp., while AP4 has not yet been able to be identified (Fig. 1). The four fungi species were also reported by Lalngaihawmi *et al.* (2018) as endophytic fungi in rice.

The presence of endophytic fungi in the roots of the Gogo rice plants varies based on the various tissues in which they grow. A study conducted by Naik *et al.* (2009) reported that the colonization of endophytic fungi in rice plants happens more dominantly at the roots of rice plants.

Pathogenicity of Endophytic Fungi isolated from *O. sativa* L. var Maninjau Seeds

Our study showed that the germination percentage of Gogo rice seeds varied (Table 1), while the invasions of endophytic fungi against the seed germination were shown in Figure 2. Each endophytic fungi showed different levels of pathogenicity, both at 7 days after inoculation and 14 days after inoculation. The level of pathogenicity is useful for determining the best isolates to be used for subsequent tests. Based on the results of pathogenicity levels of rice sprouts that grew normally, abnormally and did not grow, the best pathogenicity value was provided by *Geotrichum* sp. AP8, followed by *Penicillium* sp. AP7 and *Curvularia* sp. AP 2.

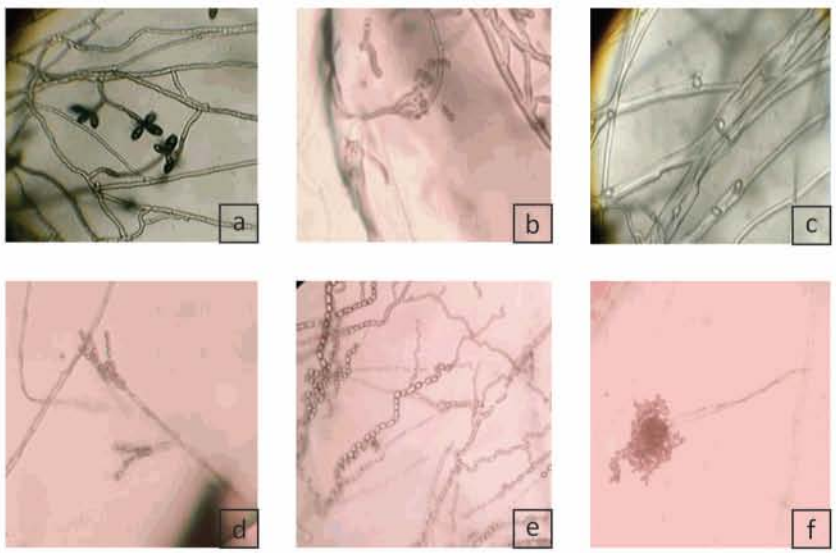

Figure 1 Microscopic characteristics of endophytic fungi isolates obtained from the roots of *O. sativa* L var. *maninjau*
Notes: a. *Curvularia* sp. AP2 (40x); b. *Penicillium* sp. AP3 (40x); c. AP4 (100x); d. *Penicillium* sp. AP7 (40x); e. *Geotrichum* sp. AP8 (40x); f. *Aspergillus* sp. AP9 (40x).

Table 1 Germination rate of the *O. sativa* L. var. *maninjau* for the 7 and 14 Days After Incubation (DAI)

Endophytic fungi	Germination (%)*					
	7-DAI			14-DAI		
	Normal	Abnormal	No growth	Normal	Abnormal	No growth
Without adding endophytic fungi	60.00 ± 8.16b	0.00 ± 0.00a	36.67 ± 4.71a	63.33 ± 9.43b	0.00 ± 0.00a	33.33 ± 4.71a
Curvularia sp. AP2	40.00±14.14a	10.00 ± 8.16a	40.00 ±8.16a	20.00 ± 8.16a	36.67 ± 12.47a	33.33 ± 4.71a
Penicillium sp. AP3	43.33 ± 4.71ab	13.33 ± 4.71a	43.33 ± 9.43a	56.67 ± 4.71ab	16.67 ± 4.71a	26.67 ± 4.71a
AP4	0.00 ± 0.00ab	0.00 ± 0.00a	100.00 ± 0.00b	6.67 ± 9.43ab	13.33 ± 12.47a	80.00 ± 14.14b
Penicillium sp. AP7	43.33 ± 9.43ab	13.33 ± 12.47a	50.00 ± 8.16a	56.67 ± 12.47ab	16.67 ± 9.43a	26.67±4.71a
Geotrichum sp. AP8	50.00 ± 8.16b	0.00 ± 0.00a	50.00 ± 8.16a	70.00 ± 8.16b	16.67 ± 4.71a	16.67±4.71a
Aspergillus sp. AP9	43.33 ± 4.71ab	6.67 ± 4.71a	50.00 ± 8.16a	46.67 ± 4.71ab	20.00 ± 0.00a	33.33±4.71a

Note : * = numbers followed by the same letter are not significantly different based on Duncan test at $P < 0.05$.

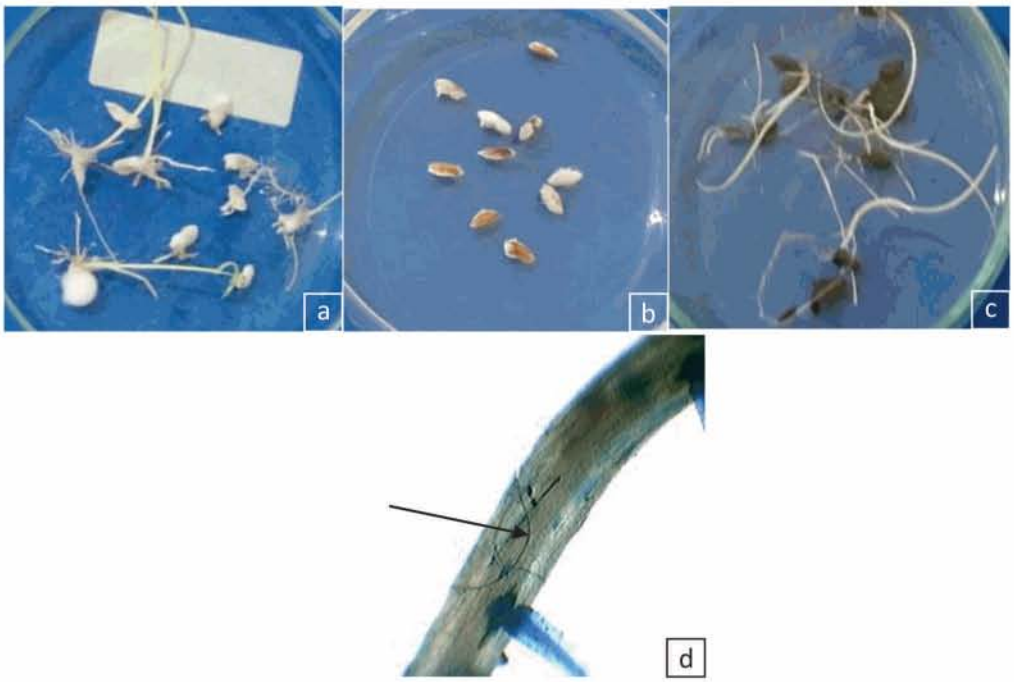

Figure 2 Rice germination with various treatments of endophytic fungi
Notes: a. rice germination without the addition of endophytic fungi; b. rice seeds with the addition of AP4 endophyte fungi (not germinated); c. rice germination with the addition of endophytic fungi *Geotrichum* sp. AP8; d. roots of rice seed undergoing treatment with endophytic fungi *Geotrichum* sp. AP8 (the arrow shows endophytic fungi invading the rice root tissue).

The results of the endophytic fungi antagonism test against *P. oryzae* showed no significant difference between the three selected fungi ($P > 0.05$) (Table 2).

Although the antagonism test did not show significant differences, the selection of the best isolate for subsequent tests was determined based on the best inhibition percentage and the diameter of the pathogen successfully inhibited. Thus, *Geotrichum* sp. AP8 was the chosen isolate. Endophytic fungi can inhibit pathogens having metabolite compounds by inhibiting the permeability of the pathogenic cells (Ting *et al.* 2011). White *et al.* (2019) added that endophytic fungi can use the mechanisms of space and nutrient competition for suppressing pathogen growth.

The Ability of Liquid Smoke and Endophytic Fungi in Inhibiting the Growth of *P. oryzae*

The results of our study indicated that liquid smoke at all tested concentrations was significantly able to inhibit the growth of pathogens compared to control (Fig 3). At liquid smoke concentrations of 0.17% to 1.75%, the inhibition percentages differed significantly ($P < 0.05$). The selection of the right liquid smoke concentration for subsequent tests is indispensable, given that liquid smoke contains several antimicrobial components that may affect not only the growth of pathogen, but also the growth of endophytic fungi.

Table 2 Diameter of *P. oryzae* colony and inhibition percentage of endophytic fungi

Endophytic fungi	Diameter of *P. oryzae* (mm)*	Inhibition percentage (%)*
Curvularia sp, AP2	41.47±2.92	21.98±5.54
Penicillium sp. AP7	32.03±7.84	41.87±14.32
Geotrichum sp. AP8	30.21±6.31	41.87±11.71

Note: * = not significantly different based on Duncan test ($P < 0.05$).

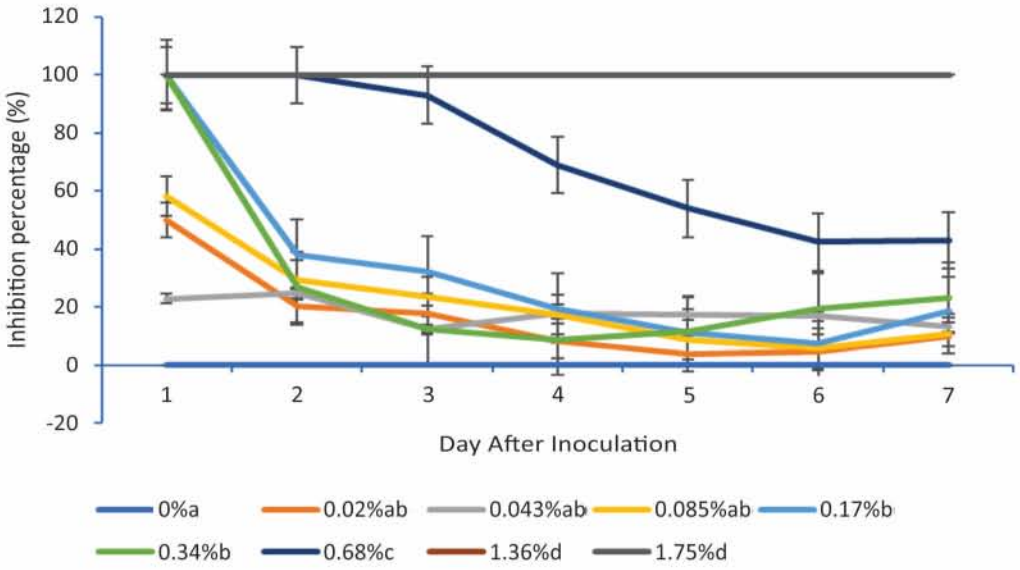

Figure 3 Inhibition percentage of different concentrations of liquid smoke against *P. oryzae* on 1 DAI until 7 DAI
Notes: The bar indicates the standard deviation. Numbers followed by the same letter are not significantly different based on Duncan test ($P < 0.05$).

The results also showed that the smallest liquid smoke concentration (0.02%) was able to inhibit the growth of pathogens despite the daily decrease in ability, while the largest liquid smoke concentration (1.75%) was able to inhibit pathogens at an inhibition percentage of 100%. Our study also indicated that the 0.17% and 0.34% liquid smoke concentrations were considered the best concentration for inhibiting pathogens compared to other concentrations. The inhibitory curve of those two concentrations had a tendency of increase after passing 4 days of inoculation, in contrast to other concentrations that had a tendency to decrease. The two liquid smoke concentrations also showed inhibiting capabilities against the tested pathogen despite the small concentrations.

The inhibiting capabilities of liquid smoke against the growth of microorganisms may have been due to the contents of active compounds originating from the pyrolysis of wood constituents (cellulose, hemicellulose, and lignin). Cellulose and hemicellulose produce organic acid compounds such as acetic acid, while lignin produces phenol compounds. The higher the content of the wood constituents, the more complex liquid smoke obtained (Pszczola 1995). Contents of active compounds in "Ulin" liquid smoke are acids, phenolics, alcohol, ketones, ethers and esters, with acetic acid as the main active compounds (71.57% of the total active compounds) (Junaidi *et al.* 2020). Liquid smoke of "Ulin" wood also contains a total acid of up to 8.88% (Junaidi *et al.* 2019), which has antimicrobial properties.

Various concentrations of selected isolate endophyte fungi filtrate (*Geotrichum* sp.) significantly inhibited the growth of *P. oryzae* compared with the control. However, there were no significant differences in inhibition percentages among concentrations (2-10%) ($P < 0.05$) (Fig. 4).

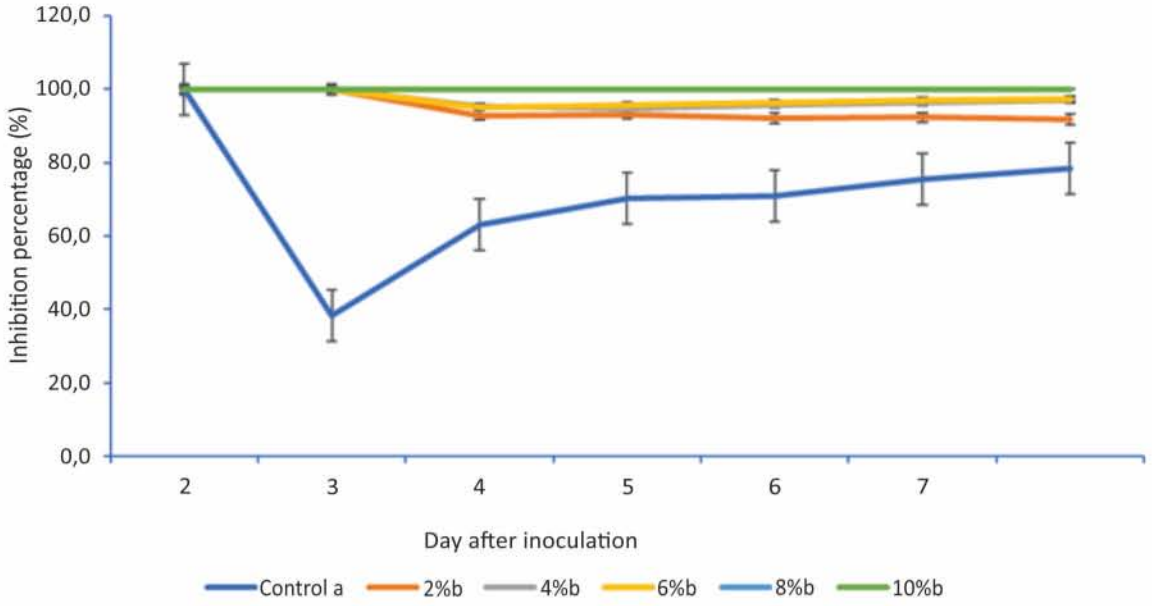

Figure 4 Inhibition percentage of *Geotrichum* sp. AP8 filtrate concentrations against *P. oryzae* on 1 DAI until 7 DAI
Notes: The bar indicates the standard deviation. Numbers followed by the same letter are not significantly different based on the Duncan test ($P < 0.05$).

Geotrichum sp. AP8 filtrate concentration of 2% was able to inhibit the growth of *P. oryzae* grown at 1 DAI up to 7 DAI with inhibition percentages ranging from 91.8 up to 100%. Therefore, the 2% concentration of *Geotrichum* sp. AP8 filtrate was chosen for testing the inhibitory synergism with liquid smoke. This result of *Geotrichum* sp. AP8 filtrate is similar to the results of previous research conducted by Imaningsih *et al.* (2021), which used endophytic fungi filtrate of "Hiyung" cayenne pepper with a concentration of 2% for inhibiting pathogen *Colletotrichum capsici* at almost 100% inhibition percentage. Our study also provides better inhibitory results for the genus *Geotrichum* compared to the study of Lalngaihawmi *et al.* (2019), which tested *Geotrichum candidum* for inhibiting *P. oryzae* with a 68% inhibition percentage at 7 DAI.

Endophytic fungi inhibit pathogen growth through anti-microbial compounds (Schulz & Boyle 2005; Singh *et al.* 2021). In our study, the filtrate of endophytic fungi was tested directly to inhibit the growth of *P. oryzae* and successfully showed high inhibition percentage. Results of our study showed that the presence of bioactive substances produced by endophytic fungi has anti-microbial properties against the tested pathogen. Singh *et al.* (2021) stated that bioactive compounds of endophytic fungi can be alkaloid, flavonoid, lignan, saponin, quinone, xanthone and miscellaneous compounds.

Synergism of Liquid Smoke and Endophytic Fungi Inhibit *P. oryzae* Growth

The growth of pathogenic *P. oryzae* was inhibited by the combination between liquid smoke concentrations of 0.17% and 0.34% and *Geotrichum* sp. AP.8 endophytic filtrate concentration of 2% with a range of inhibition percentages from 42% up to 100% on 1 DAI until 7 DAI. However, the inhibition percentages of the combination did not differ significantly among treatment combinations. The inhibition percentages of the treatments were not significantly different when compared to the control (Fig. 5). A previous study conducted by Imaningsih *et al.* (2021) showed that the concentrations combinations between liquid smoke of "Ulin" wood and endophytic fungi filtrate of "Hiyung" chili significantly inhibit the growth of pathogen *C. capsici* compared to the control.

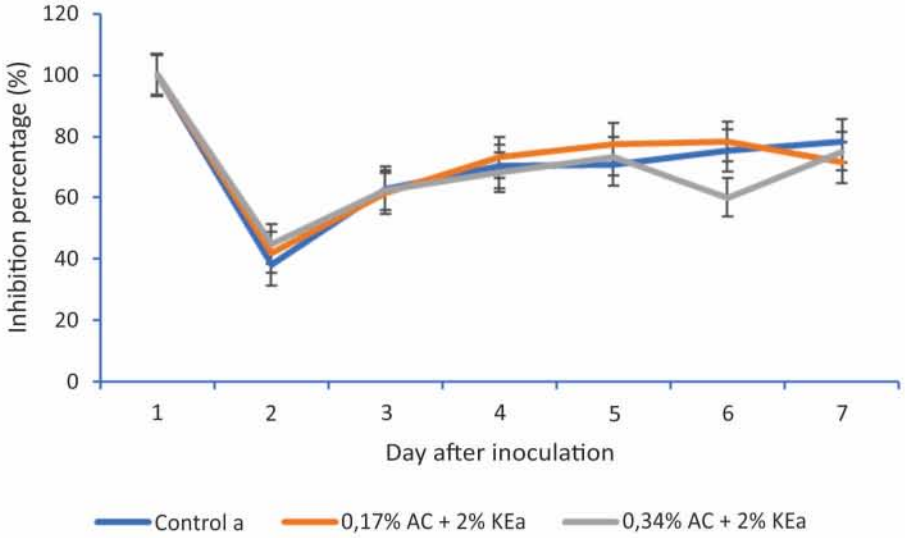

Figure 5 Inhibition percentages of several concentrations combinations between *Geotrichum* sp. AP8 filtrate (KE) and liquid smoke (AC) against *P. oryzae* on 1 DAI until 7 DAI
Notes: The bar indicates the standard deviation. Numbers followed by the same letter are not significantly different based on Duncan test ($P < 0.05$).

Based on the results of our study, the inhibition percentage achieved when combining liquid smoke and endophytic fungi filtrate was higher compared to the inhibition percentage of only using liquid smoke at the same concentration. The inhibition percentage of combining liquid smoke and endophytic fungi filtrate was lower when compared to the inhibition percentage of only using endophytic filtrate at the same concentration. During the filtration process, there might still be fungi cells carried away, due to the pore size of the filter membrane of 0.45 microns. Hyphae fragments and spores of *Geotrichum* sp. AP8 possibly penetrates the filter pores and grows during the inhibition process against the pathogens. Meanwhile, Sayer *et al.* (1969) grouped *Geotrichum* sp. into fungi with intermediate spore size, which size is smaller than the usual fungi spores size. It is suspected that the ability of endophytic fungi filtrate decreased due to the presence of the carried-away fungi cells during the filtration process. However, those carried-away fungi cells died in the presence of the liquid smoke. This was confirmed by a study conducted by Oramahi *et al.* (2011) as well as Oramahi and Yoshimura (2013) which showed that liquid smoke possesses antifungal properties because it contains phenol, carbonyl and acid compounds. In addition, acetic acid and propionate components are able to neutralize fungi cells and inhibit enzyme activity (Karseno *et al.* 2001). Therefore, in addition to inhibiting the growth of pathogenic fungi, liquid smoke is also suspected to inhibit endophytic fungi.

CONCLUSION

Liquid smoke of "Ulin" wood and endophytic fungi of "Gogo" rice var. *maninjau* has the ability to inhibit the growth of pathogen *P. oryzae*. Concentrations of 0.17% to 1.75% liquid smoke and 2% to 10% endophytic fungi filtrate showed high inhibition percentage against pathogen *P. oryzae*. The test of synergism, however, did not show an increase in inhibition percentage. Further research on the inhibitory ability and the best concentrations of liquid smoke and endophytic fungi filtrate should be conducted to provide more protection against plant pathogens, especially for rice plants.

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REFERENCES

Andriani Y. 2008. Budidaya Tanaman Padi Di Indonesia. Jakarta (ID): Sastra Hudaya.

Barnett HL, Hunter BB. 1998. Illustrated genera of imperfect fungi (4th ed.). St. Paul (US): American Phytopathological Society (APS Press). Retrieved from https://www.academia. edu/35499449/ Illustrated_genera_of_imperfect_fungi_fourth_edition_Barnett_y_Hunter_pdf_pdf.

BPPP Jateng. 2014. Kumpulan Deskripsi Varietas Padi. Semarang (ID): Balai Penelitian dan Pengembangan Pertanian.

Darmadji P. 2002. Optimasi Pemurnian Asap Cair Dengan Metode Redistilasi. J Teknologi Industri Pangan 13:267-71.

Hayashi K, Yoshida T, Hayano-Saito Y. 2019. Detection of white head symptoms of panicle blast caused by Pyricularia oryzae using cut-flower dye. Plant Methods, 15(1), p. 1-9.

Hairmansis A, Yullianida Y, Supartopo S, Suwarno S. 2017. Rice improvement for upland areas. Iptek Tanaman Pangan 11(2):95-106. Retrieved from http://www.ejurnal.litbang.pertanian.go.id/index.php/ippan/article/view/6078.

Imaningsih W, Mariana, Junaedi AB, Rasyidah. 2021. Antifungal activities of the combination of Ulin wood liquid smoke and Hiyung cayenne pepper root endophyte fungi against *Colletotrichum capsici*. Agrivita 43(1):69-78. https://doi.org/10.17503/agrivita.v1i1.2458.

Junaidi AB, Apriyani H, Abdullah, Santoso UT. 2019. Fraksinasi dan karakterisasi asap cair dari kayu ulin (*Eusideroxylon zwageri* Teijsm. & Binn.) sebagai pelarut kitosan. [Fractionation and characterization of liquid smoke from ulin wood (*Eusideroxylon zwageri* Teijsm. & Binn.) as chitosan solvent]. Jurnal Riset Industri Hasil Hutan 11(2):53-64. https://doi. org/10.24111/ jrihh.v11i2.4861.

Junaidi AB, Nursyifa A, Abdullah. 2020. Redistillation and characterization of liquid smoke from ulin wood (*Eusideroxylon zwageri* Teijsm. & Binn.) and its ability as a chitosan solvent. IOP Conference Series: Materials Science and Engineering 980(1). https://doi.org/10.1088/1757-899X/980/1/012024.

Karseno P, Darmadji., Kapti R. 2001. Daya Hambat Asap Cair Kayu Karet terhadap Bakteri Pengkontaminan Lateks dan *Ribbed Smoke Sheet*. [Rubber Wood Liquid Smoke Resistance to Latex and Ribbed Smoke Sheet Bacteria]. Agritect 21(1):10-5.

Lalngaihawmi, Banik S, Chakruno P. 2019. Isolation of fungal endophytes of rice and their antagonistic effect against some important rice fungal pathogens *in vitro*. J Pharmacon Phytochem 8(4):649-53. www. phytojurnal.com.

Lee SH, H'ng PS, Cow MJ, Sajap AS, Tey BT, Salmiah U, Sun YL. 2011. Effectiveness of Pyroligeneous Acid from Vapour Released in Charcoal Industry Against Biological Attacks Under Laboratory Condition. J Appl Sci 11(24):3848-53.

Luqman, Rizalinda, Khotimah S. 2015. Jamur Mikoriza Vesikular Arbuskular (MVA) pada Rhizosfer Tanaman Langsat (*Lansium domesticum* Corr.) di Lahan Gambut. [Vesicular Arbuscular Micorhyzae (VAM) from *Lansium domesticum* Corr. Rhizosphere at Swamp Land]. Protobiont 4(3):89-97.

Manurung IR, Pinem MI, Lubis L. 2014. Uji Antagonism Jamur Endofit terhadap *Cercospora oryzae* Miyake dan *Culvularia lunata* (wakk) Boed. dari Tanaman Padi di Laboratorium. [Endophyte Mushroom Antagonism Test Against *Cercospora oryzae* Miyake and *Culvularia lunata* (Wakk) Boed. from Rice Plants in the Laboratory]. Jurnal Online Agroekoteknologi. 2(4):1563-71.

Naik BS, Shashikala J, Krishnamurthy YL. 2009. Study on the Diversity of Endophytic Communities from Rice (*Oryza sativa* L.) and Their Antagonistic Activities *in vitro*. J. Microbiol Res 164:290-6.

Norsalis E. 2011. Padi Gogo dan Sawah. [Gogo Rice and Field Rice]. Jurnal Online Agroekoteknologi 1(2):1-6.

Nurzannah SE, Lisnawita, Bakti D. 2014. Potensi jamur endofit asal cabai sebagai agens hayati untuk mengendalikan layu fusarium (*Fusarium oxysporum*) pada cabai dan interaksinya. [The potential of endophytic fungi from chili as biological agents to control fusarium withers (*Fusarium oxysporum*) in chili peppers and their interactions]. Jurnal Online Agroteknologi 2(3):1230-8.

Oramahi HA, Yoshimura T. 2013. Antifungal and Antitermitic Activities of Wood Vinegar from *Vitex pubescens* Vahl. J Wood Sci 59:344-50.

Oramahi HA, Diba F, Wahdina. 2011. Aktivitas Antijamur Asap Cair dari Serbuk Gergaji Kayu Akasia (*Acacia mangium* Willd) dan Kayu Laban (*Vitex pubescens* Vahl). [Liquid Smoke Antifungal Activity of Acacia Sawdust (*Acacia mangium* Willd) and Laban Wood (*Vitex pubescens* Vahl]. Bionatura. Jurnal Ilmu-Ilmu Hayati dan Fisik 13(1):79-84. ISSN 1411-0903.

Pszczola DE. 1995. Tour highlights production and uses of smoke-based flavors. Food Technology 49(1):70-4.

Rabha AJ, Naglot A, Sharma GD, Gogoi HK, & Veer V. 2014. *In Vitro* Evaluation of Antagonism of Endophytic *Colletotrichum gloeosporioides* Against Potent Fungal Pathogens of *Camellia sinensis*. Indian J. of Microbiol., 54(3), p. 302–9.

Sayer WJ, Shean DB, Ghosseiri J. 1969. Estimation of airborne fungal flora by the Andersen sampler versus the gravity settling culture plate. I. Isolation frequency and numbers of colonies. Allergy 44(4):214-27. https://doi.org/10.1016/0021-8707 (69)90088-4.

Schulz B, Boyle C. 2005. The endophytic continuum. Mycol Res 109: 661-87.

Soldera S, Sebastianutto N, Bortokmenzzi R. 2008. Composition of Phenolic Compounds and Antioxidant Activity of Commercial Aqueous Smoke Flavorings. J Agric Food Chem 56:2727-34.

Singh A, Singh DK, Kharwar RN, White JF, Gond SK. 2021. Fungal endophytes as efficient sources of plant-derived bioactive compounds and their prospective applications in natural product drug discovery: Insights, avenues, and challenges. Microorganisms9(1):1-42. https://doi.org/10. 3390/microorganisms9010197.

Suganda T, Yulia E., Widiantini F, Hersanti. 2016. Intensitas Penyakit Blas (*Pyricularia oryzae* Cav.) pada Padi Varietas Ciherang di Lokasi Endemik dan Pengaruhnya terhadap Kehilangan Hasil.[Intensity of Blas Disease (*Pyricularia oryzae* Cav.) in Ciherang Rice Varieties in Endemic Locations and Their Effect on Loss of Yield]. Jurnal Agrikultura 27(3):154-9.

Talukdar D. 2011. Effect of arsenic-induced toxicity on morphological traits of *Trigonella foenum-graecum* L. and *Lathyrus sativus* L. during germination and early seedling growth. Curr Res J Biol Sci 2(3):116-23.

Ting ASY, Mah SW, Tee CS. 2010. Identification of Volatile Metabolites from Fungal Endophytes with Biocontrol Potential towards *Fusarium oxysporum* F. sp. *cubense* Race 4. Am J Agric Biol Sci 5(2):177-82.

Tomah AA, Abd Alamer IS, Li B, Zhang JZ. 2020. A new species of *Trichoderma* and gliotoxin role: A new observation in enhancing biocontrol potential of *T. virens* against *Phytophthora capsici* on chili pepper. Biol Control 145:104261. https://doi.org/ 10.1016/j. biocontrol.2020. 104261.

Wang X, Lee S, Wang J, Ma J, Bianco T, Jia Y. 2014. Current advances on genetic resistance to rice blast disease. Chapter 7 in Rice-Germplasm, Genetics and Improvement (Yan W & Bao J, Eds.). Available at: http://www.intechopen.com/ books/rice-germplasm-genetics-and-improvement /current-advances-on-genetic-resistance-to-rice blast-disease. [Internet] [accessed on 30 November 2018].

Waruwu AAS, Soekarno BPW, Munif A. 2016. Metabolit cendawan endofit tanaman padi sebagai alternatif pengendalian cendawan pathogen terbawa benih padi. [Metabolite endophytic mushrooms of rice plants as an alternative to control pathogenic mushrooms carried by rice seeds]. Jurnal Fitopatologi Indonesia. 12(2):53-61.

White JF, Kingsley KL, Zhang Q, Verma R, Obi N, Dvinskikh S, …, Kowalski KP. 2019. Review: Endophytic microbes and their potential applications in crop management. Pest Manag Sci 75(10):2558-65. https://doi.org/10.1002/ps.5527.

CHARACTERIZATION AND POLYPHASIC IDENTIFICATION OF NOVEL RHIZOBACTERIA STRAIN ISOLATED FROM SAND DUNES ECOSYSTEM

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ABSTRACT

The coastal sand dune ecosystem at the Parangtritis Coast of Yogyakarta, Indonesia has unique characteristics such as low moisture sandy soil, high salinity and low nutrient content. *Fimbristylis cymosa* is one of the plant species having the capability to survive in that unique ecosystem. In this study, rhizobacteria isolated from the rhizosphere of *F. cymosa* were isolated to be further analyzed on their phosphate solubilizing and antagonistic properties against *Fusarium oxysporum* which cause the Wilt disease. The isolates of Phosphate Solubilizing Rhizobacteria (PSR) having the most potential capabilities were then polyphasically identified based on phenotypic and genotypic characters followed by 16S rDNA sequencing. The results showed that four PSR isolates (I8, I11, I12 and I24) have high phosphate dissolution indices. The highest indices were observed in isolates I11 (3.08) and I12 (3.44), respectively. Analysis of the dual plate experiments for PSR I11 and PSR I12 isolates against the growth of *F. oxysporum* also showed quite high inhibitory activities, i.e., isolate PSR I11 was 42.40%, while isolate PSR I12 was 42.08%. The two isolates were polyphasically identified as *Burkholderia dolosa*. This study clearly showed that PSR I11 and PSR I12 isolates are very potential and prospective to be used as marginal land inoculants and as providers of phosphorus. This study also showed that the isolates are useful as biocontrol agents against *F. oxysporum* in plants.

Keywords: inhibitory activity, phosphate dissolution index, phosphorus, polyphasic identification, sandy soil

INTRODUCTION

Coastal sand dunes are Aeolian landforms commonly found in coastal areas at all latitudes in the world. Indonesia has coastal sand dunes located in the southern part of Java Island, extending from the southern coast of West Java Province to Yogyakarta Province. The most significant sand dunes formation occurs in the Parangtritis Coastal Area, located on the southern coast of Yogyakarta Province. The sand dunes ecosystem has a high temperature, relatively little vegetation, strong winds, high salt content and very low groundwater content, making it a dry and infertile area (Mahdavi & Bergmeier 2016; Campos *et al.* 2020). In general, plants are difficult to grow in dry and less fertile areas. However, there are several groups of plants that can survive in the sand dunes habitat. Among plants that survived the sand dunes habitat on Yogyakarta's southern coast is *Fimbristylis cymosa*, a family of Cyperaceae, which grows well and is widespread along with the coastal sand dunes habitat.

The growth and development of *F. cymosa* in the coastal sand dunes habitat are strongly influenced by the availability of essential soil macronutrients. One of the main macronutrients supporting the growth and development of *F. cymosa* is phosphorus. Phosphorus is among minerals needed at 0.2% dry weight of *F. cymosa*, as a component of nucleic acids (DNA and RNA) for conserving energy. Plants absorb phosphorus in the form of phosphate anions (PO_4^- or PO_4^{2-}) which are dissolved in groundwater. However, phosphate anions have

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a high tendency to bind with calcium, aluminum, and iron in the soil, which makes them insoluble and easily settled so that they cannot be absorbed by plants, including *F. cymosa* (Yang *et al.* 2021). Undissolved phosphorus in the soil can be dissolved by the Phosphate Solubilizing Bacteria (PSB) group.

A study conducted by Liu *et al.* (2015) in calcareous soil found PSB genera *Bacillus, Pseudomonas, Rhizobium* and *Acinetobacter.* Pastore *et al.* (2020) reported PSB genera *Burkholderia, Bacillus*, and *Pseudomonas* in forest soils. In the mangrove forest rhizosphere, Teymouri *et al.* (2016) observed the existence of PSB genera *Bacillus, Pseudomonas* and *Acinetobacter.* Rasul *et al.* (2019) obtained PSB genera *Pseudomonas* and *Bacillus* from rhizospheric paddy field soil, while the PSB genus *Paenibacillus* was found from wheat rhizosphere (Cherchali *et al.* 2019).

In soil environments, there are also soilborne pathogens causing plants infection. One species of a soil-borne pathogen is *Fusarium oxysporum* causing the *Fusarium* Wilt disease in various types of agricultural crops within the Solanaceae family, such as tomatoes, potatoes, and chilies (Ávila *et al.* 2019; Srinivas *et al.* 2019; Chowdhury *et al.* 2020). The wilting phenomenon that occurred in those crops due to *Fusarium* Wilt disease resulted in a significant reduction in crop yields and has been a major problem for agriculture worldwide. The use of chemical compounds for treating the Wilt disease has adverse effects on non-target organisms. Therefore, it is pertinent to find biocontrol agents which is considered safer for the environment.

In soil environments, Phosphate Solubilizing Bacteria (PSB) are reported to be more dominantly existing in the rhizosphere. In addition, PSB from the rhizosphere is known to produce active metabolites compared to other sources and is able to increase plant growth and development. PSB originating from extreme environments such as sand dunes, might have specific characteristics as a biocontrol agent against *F. oxysporum* (Ahluwalia *et al.* 2021; Rasool *et al.* 2021) Therefore, this study aimed to: 1. isolate and characterize Phosphate Solubilizing Bacteria (PSB) from the rhizosphere of *F. cymosa* living in sand dunes habitat; and 2. screen the capability of isolated PSB as a biocontrol agent against *F. oxysporum.*

MATERIALS AND METHODS

Rhizosphere Soil Samples and Isolate of *F. oxysporum*

Rhizosphere soil samples from the planting area of *F. cymosa* were taken and collected from 8 different sampling points along with the coastal sand dune habitat in the Parangtritis Coastal Area, Yogyakarta. The roots of the *F. cymosa* were carefully extracted with a shovel. Soil attached to the roots was collected in sterile plastic containers as rhizosphere soil samples. The samples were then stored at 4 °C for further isolation of Phosphate Solubilizing Bacteria (PSB).

The isolate of *Fusarium oxysporum* was obtained from the culture collection of the Microbiology Laboratory, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia and was grown in Potato Dextrose Agar (PDA) medium at 30 °C for 10 days.

PSB Isolation and Phosphate Dissolution Index Analysis

Ten (10) g of rhizosphere soil samples were poured into a 50 mL Erlenmeyer and suspended in 90 mL of sterile distilled water, then shaken using a vortex at a speed of 200 rpm for 30 min. The sample suspension was diluted in a series of up to 10^{-4} dilutions. A total of 0.1 mL of the respective 10^{-3} and 10^{-4} diluted sample suspensions was inoculated on the National Botanical Research Institute's Phosphate (NBRIP) agar medium by means of the spread plate method, then incubated at 30 °C for 48 - 72 hours. The NBRIP medium consists of 1% glucose, 0.5% $Ca_3(PO_4)_2$, 0.5% $MgCl_2$, 0.01% $(NH_4)_2SO_4$, 0.0.5% $MgSO_4.7H_2O$, and 0.02% KCl (Nautiyal 1999). PSB isolates having a clear zone around the colony were then re-isolated as PSR, and purified on an NBRIP medium.

In order to measure the phosphate dissolution index, the PSR isolates were re-cultured in NBRIP liquid medium and incubated at 30 °C for 48 h. Forty (40) µL of the incubated culture was diluted until reaching a colony having cell number 10^8 CFU/mL. The liquid culture was then poured into a Petri dish containing NBRIP agar medium by means of the pour plate method and incubated at 30 °C for 48 h.

The formed clear zone and the size of the bacterial colony were calculated. The phosphate dissolution index was determined with the following formula developed by Haile *et al.* (2016):

$$PSI = \frac{(HZD + CD)}{CD}$$

where:
PSI = Phosphate Solubilization Index
HZD = Halo Zone Diameter (mm)
CD = Colony Diameter (mm)

Antagonistic Activity of PSR Against *F. oxysporum*

In vitro test of PSR antagonist activity against *F. oxysporum* was carried out by using the dual plate experiments method according to Zhang *et al.* (2017) with minor modifications. Potato Dextrose Agar (PDA) medium was prepared on a 9 cm diameter Petri dish, followed by making a 5-mm diameter well in the PDA medium at the center of the Petri dish by using a cork borer. The other four 5-mm diameter wells were made at a distance of 2 cm from the well located at the center of the Petri dish and from each other. *F. oxysporum* mycelium with a diameter of 5 mm was taken from the outermost part of the 10-day-old fungal colony and was inoculated into the well at the center of the Petri dish containing PDA medium by using an inoculation needle. A total of 8 µL of PSR isolate suspension with a cell number of 10^9 CFU/mL was inoculated into the other four wells in the same Petri dish and then incubated at 30 °C for 10 days. The control agar plates were then prepared with the same agar medium without inoculating the PSR isolates suspension. At the end of the incubation time, the diameter of *F. oxysporum* colonies was measured diagonally, vertically and horizontally. The inhibition percentage of PSR isolates against *F. oxysporum* was determined using the following formula of Royse and Ries (1978):

$$IP = \frac{(R1 - R2)}{R1} \times 100\%$$

where:
IP = Inhibition Percentage
R1 = Diameter of *F. oxysporum* colonies on the control plate
R2 = Diameter of *F. oxysporum* colonies on the treated plate

Polyphasic Identification of PSR Isolates

Polyphasic identification of PSR isolates was determined based on a combination of phenotypic and genotypic characters. Phenotypic characters of the observed isolates, including the morphological and biochemical characters, were determined by referring to Bergey's Manual of Systematic Bacteriology (Brenner *et al.* 2005). The morphological characters were observed based on cell and colony morphologies. The cell morphology of PSB isolates was observed based on the shape and characteristics resulting from the gram staining procedure. Meanwhile, colony morphology of PSB isolates was observed based on the shape, color, margins, elevation, internal structure and optical features of the colony. The biochemical characters were determined based on several tests, such as the motility, catalase, oxidase tests, glucose, sucrose, lactose fermentation tests, urease and Voges-Proskauer (VP) tests.

The isolates were then characterized genotypically based on the 16S rDNA nucleotide sequence. Total genomic DNA of PSB isolates was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA). PCR amplification of the 16S rDNA gene of the PSB isolates was analyzed by using 27F primers (5'-AGAGTTTGATCCT GGCTCAG-3') and 1492R (5'- GGTTACCTT GTTACGACTT-3') (Hossain *et al.* 2020). The PCR amplification was performed using a DNA thermal cycler (Bio-Rad, USA) with the following program: pre-denaturation at 95 °C for 30 min, followed by 30 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 57 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR products were purified and sequenced. The nucleotide sequences were then compared with DNA sequence databases (GenBank) through the BLAST program (BLASTn) at the National Biotechnology Information Center (NCBI). A phylogenetic tree was constructed through the maximum likelihood method using Molecular Evolutionary Genetics Analysis X (MEGA version X).

Statistical Analysis

Data were statistically analyzed using the one-way variance analysis (ANOVA) followed by the Duncan Multiple Range Test (DMRT) approach for testing the mean differences. At $P < 0.05$,

the differences were considered significant. Results are expressed as means of three replicates ± standard deviation. The software used was IBM SPSS Statistics version 21.

RESULTS AND DISCUSSION

PSR Isolate and Phosphate Solubilization Index

A total of 26 bacteria were isolated from *F. cymosa* rhizosphere soil samples in the sand dunes habitat of Parangtritis Coastal Area, Yogyakarta, Indonesia. Of the 26 rhizobacterial isolates, 4 rhizobacterial isolates were selected as PSR based on the clear halo formation around the colony in the NBRIP medium containing tricalcium phosphate. The clear halo was formed due to the production of organic acids or polysaccharides as a result of the phosphatase enzyme activity of the PSR isolate (Paul & Sinha 2013; Behera *et al.* 2017). The phosphate solubilization indices of each PSR isolate after 10 days of incubation are presented in Table 1 (Widane *et al.* 2018).

The highest phosphate solubilization index of 3.44 was obtained from PSB I12. Meanwhile, the lowest phosphate solubilization index of 3.00 was obtained from PSB I24. A previous study by Teymouri *et al.* (2016) reported the phosphate solubilization index of 3.5 which was obtained from *Bacillus* sp. found in the rhizosphere of mangrove forest. Mardad *et al.* (2013) reported the phosphate solubilization index of 3.5 which was obtained from *Enterobacter hormaechei* found in the phosphate rock deposits. In contrast, several other researchers reported a lower-than-three phosphate solubilization index, such as those obtained from *Pseudomonas* (2.6) and *Acinetobacter* (2.0) found in the rhizosphere of forest (Teymouri *et al.* 2016). The *Paenibacillus* genus from the wheat rhizosphere was reported to have a very low phosphate solubilization index of 1.32 (Cherchali *et al.* 2019). *Pseudomonas* and *Serratia* obtained from the rhizosphere of *Allium cepa* L. were also reported to have low phosphate solubilization indices of 2.0 and 2.1, respectively (Blanco-Vargas *et al.* 2020).

Table 1 Phosphate solubilization index of PSR isolates obtained from the *F. cymosa* rhizosphere after 10 days of incubation

No	PSR Isolate	Phosphate solubilization index
1	I8	3.04 ± 0.51^{bcd}
2	I11	3.08 ± 0.38^{cd}
3	I12	3.44 ± 0.19^{d}
4	I24	3.00 ± 0.43^{abcd}

Notes: The index is presented as mean ± SD; Numbers followed by the same letter in a column do not show a significant difference according to the Duncan test (DMRT) at $P < 0.05$.

Different values of phosphate solubilization index among PSR isolates are closely related to organic acids production, causing a pH decrease in bacterial cells and their environment, leading to proton substitution in the phosphate mineral and P release (Ludueña *et al.* 2018; Rasul *et al.* 2021). Organic acids produced by several PSRs include gluconic, glycolic, oxalic, malonic and succinic acids. Gluconic acid is an organic acid having a role as a phosphate solvent (Joe *et al.* 2018; Santos-Torres *et al.* 2021), which is useful in providing P minerals needed for plant growth (Valetti *et al.* 2018; Sahandi *et al.* 2019). PSR I11 and PSR I12 isolates have a high ability to dissolve phosphate *in vitro*. Therefore, these two isolates are the potential to be developed as agents for providing P for plants.

Antagonistic Activity of PSR Isolates Against *F. oxysporum*

PSR I8, PSR I11, PSR I12, and PSR I24 isolates were tested *in-vitro* for their antagonistic activity against *F. oxysporum* by using the dual plate technique. The results showed a significant inhibitory effect on the growth of *F. oxysporum* mycelium (Fig. 1), which was clearly seen by comparing the inhibitory effect of the four isolates with the control against the growth of *F. oxysporum*. The four PSR isolates clearly caused a smaller colony size of *F. oxysporum* compared to that in the control treatment. The colony diameter of *F. oxysporum*, however, was different among the four PSR isolates, i.e., 44.75 mm was observed in PSR I11 isolate, 45.00 mm in PSR I12, 53.00 mm in PSR I24 and 62.50 mm in PSR I8 (Widane *et al.* 2018).

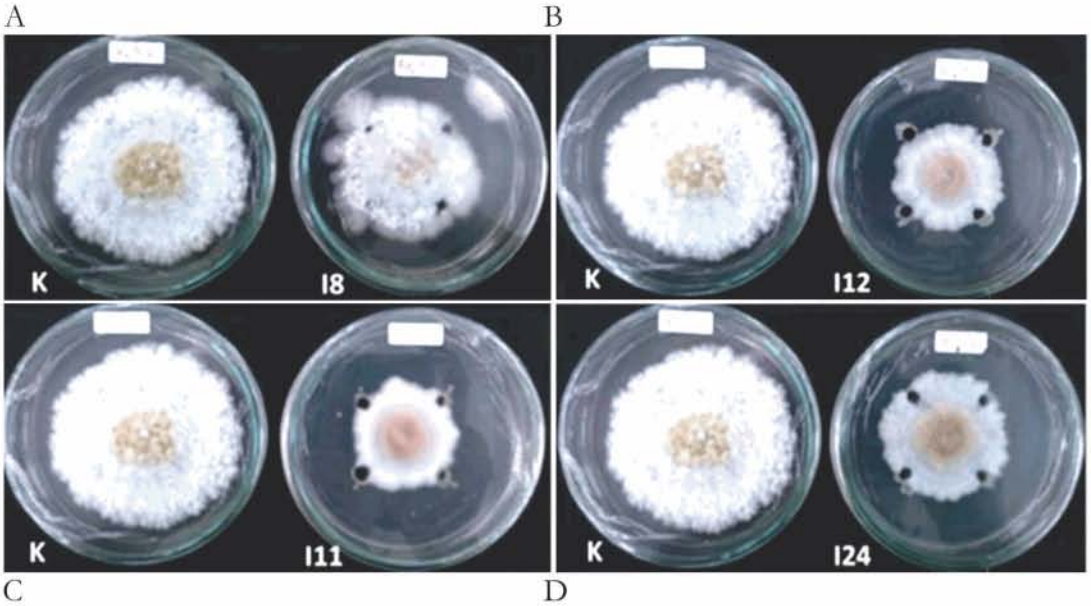

Figure 1 Antagonistic activity of PSR isolates against *F. oxysporum* observed *in-vitro* after 14 days incubation period
Notes: A = PSR I8; B = PSR I12; C = PSR I11; D = PSR I24.

In addition, our study showed that PSR isolates also caused thinner *F. oxysporum* mycelium growth compared to that in the control treatment. This explains the inability of *F. oxysporum* to grow well in the presence of PSR isolates. The graph of the inhibition percentage of PSR isolates against *F. oxysporum* is presented in Figure 2 (Widane *et al.* 2018).

The dual culture method applied in this study allows direct *in-vitro* interactions between PSR isolates and *F. oxysporum*. These interactions can be in the form of competition for space or for nutrients from the growth medium, leading to growth inhibition of *F. oxysporum*. Growth inhibition can also be caused by metabolite compounds produced by PSR isolates. Several metabolite compounds are reported to have the most effective antagonistic activity in inhibiting the growth of plant pathogens in the form of antibiotics, siderophores and bacteriocins (Khedhera *et al.* 2021). Bacteria can produce hydrolytic enzymes such as protease, lipase, chitinase and glucanase which can lyse fungal cells so that they have antagonistic activity against plant pathogenic fungi (Cui *et al.* 2019; Lau *et al.* 2020).

Our study also showed that the PSR I11 isolate was the most effective at inhibiting the growth of *F. oxysporum* with an inhibition percentage of 42.40%, while the PSR I12 isolate had an inhibition percentage of 42.08% which is not significantly different from that of PSR I11. Lower inhibition percentages were observed for PSR I24 and PSR I8, i.e., 31.82% and 19.58%, respectively (Fig. 2).

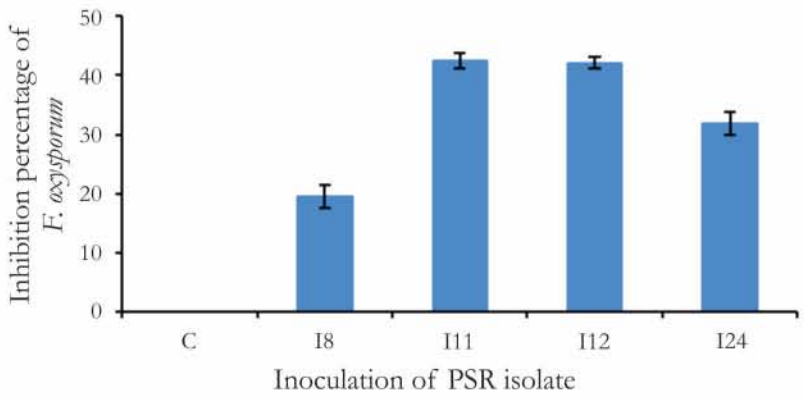

Figure 2 Inhibition percentage value of PSR isolates against *F. oxysporum* after 14 days of incubation period

Several studies have also reported that bacteria isolated from the rhizosphere are able to protect plants from fungal pathogens (Babu *et al.* 2015; Panda *et al.* 2016). *Bacillus amyloliquefaciens* from the rhizosphere of apple trees were reported to have inhibition capability against *F. oxysporum* with an inhibition percentage of 35% (Guleria *et al.* 2016). Meanwhile, research conducted by Jangir *et al.* (2018) showed that *Bacillus* sp. from the rhizosphere of tomato plants had antagonistic activity against *F. oxysporum* with higher inhibition percentage (70%) and therefore, is potential as a biocontrol agent against *Fusarium* Wilt disease in tomato plants. Karthika *et al.* (2020) also isolated *Bacillus cereus* from the rhizosphere of a tomato plant, which has an inhibition percentage of 66%. Our study further showed that PSR I11 and PSR I12 isolates have the capability of phosphate solvent and have high antagonistic activity against *F. oxysporum.*

Polyphasic Identification of PSR Isolates

Observation of the morphological characters of PSR I11 and PSR I12 showed different colony morphology forms, i.e., circular and irregular, respectively (Table 2). However, the two isolates have the same colony color, elevation, internal structure, optical features and shape, such as cream, entire, convex, smooth, translucent and rod-shaped (Table 2).

Biochemical properties of the two isolates, PSR I11 and PSR I12 showed similarities in terms of motility, production of catalase and oxidase enzymes and positive reaction toward the Voges-Proskauer test. Different reactions occurred when being tested in a medium containing urea, showing that PSR I11 isolate is able to produce urease, while PSR I12 isolate is not able to produce urease (Table 3).

Comparing the results from the identification of the morphological and biochemical characters with the characters described in Bergey's Manual of Determinative Bacteriology, it is concluded that PSR I11 and PSR I12 isolates belong to the genus *Burkholderia* (Brenner *et al.* 2005).

Table 2 Morphological characters of PSR isolates from rhizosphere of *F. cymosa*

No	Morphological characters		PSR isolate	
			I11	I12
1.	Colony morphology	Shape	Circular	Irregular
		Color	Cream	Cream
		Margin	Entire	Entire
		Elevation	Convex	Convex
		Internal structure	Smooth	Smooth
		Optical feature	Translucent	Translucent
2.	Cell morphology	Shape	Rod-shaped	Rod-shaped
		Gram properties	Negative	Negative

Tabel 3 Biochemical characters of two PSR isolates from rhizosphere of *F. cymosa*

No	Biochemical characters	PSR isolate	
		I11	I12
1	Motility test	+	+
2	Catalase test	+	+
3	Oxidase test	+	+
4	Glucose fermentation	-	-
5	Sucrose fermentation	-	-
6	Lactose fermentation	-	-
7	Urease	+	-
8	Voges-proskauer (VP) test	+	+

Burkholderia spp. is reported of having the ability to dissolve phosphate and at the same time has antagonistic activity against *F. oxysporum*. Simonetti *et al.* (2018) isolated *Burkholderia ambifaria* from the rhizosphere of barley having a role as phosphate solvents and antagonistic properties against *F. oxysporum*. *Burkholderia cepacia* and *Burkholderia contaminans* with similar capabilities were isolated from the rhizosphere of maize (Zhao *et al.* 2014; Tagele *et al.* 2018). *Burkholderia* sp. was also obtained from the rhizosphere of Juçara palm (*Euterpe edulis* Mart) (de Castilho *et al.* 2020). In addition to the plant rhizosphere, *Burkholderia* spp. with the same properties is also obtained from the root nodules of fenugreek (*Trigonella foenum-graecum* L.) (Kumar *et al.* 2017). *B. contaminans* were also existed at the nodule of the common bean (*Phaseolus vulgaris*) (Tapia-García *et al.* 2020). Apart from dissolving phosphate and having antagonistic properties against *F. oxysporum*, *B. contaminans* also has nitrogenase properties (Silva *et al.* 2012).

Both PSR isolates were also genotypically identified using the 16S rDNA genetic marker. Based on the 16S rDNA gene sequencing, the PSR I11 and PSR I12 isolates had similarities with *Burkholderia dolosa* strain LMG 18943B with a similarity index of 99.51% and 99.44%, respectively (Table 4). The two PSR isolates were categorized in the same species because the similarity percentage in the 16S rRNA gene sequences was both ≥ 99% (Schlaberg *et al.* 2012). Phylogenetic trees based on 16S rDNA sequences of PSR I11 and PSR I12 isolates were reconstructed by using comparative sequences with the in-group and out-group categories obtained from NCBI (Table 4).

Some of the relatively close sequences of *Burkholderia* spp. included *Burkholderia dolosa* strain LMG 18943 (Yarza *et al.* 2013), *Burkholderia latens* strain R-563 (Vanlaere *et al.* 2008), *Burkholderia multivorans* strain Struelens (Bauernfeind *et al.* 1999), *Burkholderia vietnamiensis* strain LMG 10929 (LiPuma *et al.* 1999), *Burkholderia vietnamiensis* strain TVV75 (Viallard *et al.* 1998), *Burkholderia metallica* strain R-16017 (Vanlaere *et al.* 2008), *Burkholderia territorii* strain LMG 28158 (De Smet *et al.* 2015) and *Burkholderia seminalis* strain R-2419 (Vanlaere *et al.* 2008) as the in-group. Meanwhile, *Alcaligenes faecalis* subsp. *parafaecalis* strain G 16S was used as the out-group because the species is a class-level classification group Betaproteobacteria with *Burkholderia* spp. In addition, *Alcaligenes* sp. has the ability to dissolve phosphate and antibiosis properties against *F. oxysporum* (Rasool *et al.* 2021). Reconstruction of the phylogenetic tree was carried out using the maximum likelihood (ML) method with a substitution model (Tamura-Nei 93, G: gamma-distributed) and a bootstrap of 1,000 replications (Fig. 3). This phylogenetic tree shows that both strains form clusters with *Burkholderia dolosa* strain LMG 18943 in 83% bootstrap replications; therefore, reconstruction of these phylogenic trees can be trusted (Hillis & Bull 1993). The phylogenetic tree reconstruction of the two isolates was also in accordance with the BLAST results, which indicated that the two isolates could be identified as *Burkholderia dolosa*. In addition, we already submitted the 16S rDNA sequences of both strains (*B. dolosa* I11 and *B. dolosa* I12) to the GenBank, with accession numbers of OK083732 and OK083731, respectively https://www.ncbi.nlm.nih.gov).

Tabel 4 Identify sequences of PSR isolates from the rhizosphere of *F. cymosa* based on the GenBank data by using BLAST

Isolate	Accession number	Species of PSR homolog	Identity	Query cover	Reference
PSR I11	NR_104973.1	*Burkholderia dolosa* strain LMG 18943	99.51 %	99%	Yarza *et al.* (2013)
	NR_042632.1	*Burkholderia latens* strain R-5630	99.30 %	99%	Vanlaere *et al.* (2008)
	NR_029358.1	*Burkholderia multivorans* strain Struelens	99.09 %	99%	Bauernfeind et al. (1999)
	NR_041720.1	*Burkholderia vietnamiensis* strain LMG 10929	99.09 %	99%	LiPuma *et al.* (1999)
	NR_118872.1	*Burkholderia vietnamiensis* strain TVV75	99.22%	98%	Viallard *et al.* (1998)
	NR_042636.1	*Burkholderia metallica* strain R-16017	98.95%	99%	Vanlaere *et al.* (2008)
	NR_136496.1	*Burkholderia territorii* strain LMG 28158	98.95%	99%	De Smet *et al.* (2015)
	NR_042635.1	*Burkholderia seminalis* strain R-2419	98.74%	99%	Vanlaere *et al.* (2008)
PSR I12	NR_104973.1	*Burkholderia dolosa* strain LMG 18943	99.44 %	99%	Yarza *et al.* (2013)
	NR_042632.1	*Burkholderia latens* strain R-5630	99.16 %	99%	Vanlaere *et al.* (2008)
	NR_029358.1	*Burkholderia multivorans* strain Struelens	99.09 %	99%	Bauernfeind *et al.* (1999)
	NR_041720.1	*Burkholderia vietnamiensis* strain LMG 10929	99.09 %	99%	LiPuma *et al.* (1999)
	NR_118872.1	*Burkholderia vietnamiensis* strain TVV75	99.43%	98%	Viallard *et al.* (1998)
	NR_042636.1	*Burkholderia metallica* strain R-16017	98.95%	99%	Vanlaere *et al.* (2008)
	NR_136496.1	*Burkholderia territorii* strain LMG 28158	98.88%	99%	De Smet *et al.* (2015)
	NR_042635.1	*Burkholderia seminalis* strain R-2419	98.81%	99%	Vanlaere *et al.* (2008)

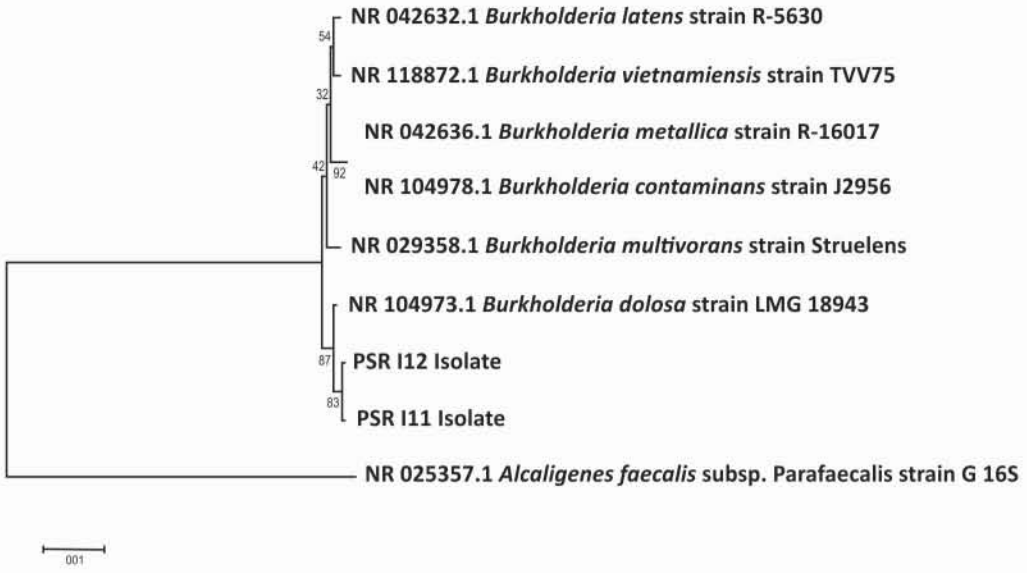

Figure 3 Phylogenetic tree based on 16S rDNA sequences constructed by the maximum likelihood method
Note: Numerals at the nodes indicate the bootstrap value (%) derived from 1,000 replication.

CONCLUSION

PSR I11 and PSR I12 bacterial isolates obtained from the rhizosphere of *F. cymosa* in the sand dunes habitat of Parangtritis, Yogyakarta, Indonesia showed the capability to dissolve phosphate. The two isolates also showed an inhibition percentage of more than 40%, and therefore, can be used as biocontrol agents against *F. oxysporum*. PSR I11 and PSR I12 isolates were polyphasically identified as *Burkholderia dolosa*. Since the two isolates were obtained from *F. cymosa* living in sand dunes habitat, which is an extreme environment, this study can be further developed into determining the possibility of obtaining these two isolates in other marginal lands, to be used as phosphate solvent and biocontrol agents against *F. oxysporum*. Further research is also suggested to determine the ability of these two isolates for procuring phosphorous mineral supply and as biocontrol agents against *F. oxysporum* in plants, especially Solanaceae family, plants both in polybags and agricultural land.

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REFERENCES

Ahluwalia O, Singh PC, Bhatia R. 2021. A review on drought stress in plants: Implications, mitigation and the role of plant growth promoting rhizobacteria. Res Environ Sustain 5:100032.

Ávila AC, Ochoa J, Proaño K, Martínez MC. 2019. Jasmonic acid and nitric oxide protects naranjilla (*Solanum quitoense*) against infection by *Fusarium oxysporum* f. sp. *quitoense* by eliciting plant defense responses. Physiol Mol Plant Pathol 106:129-36.

Babu AN, Jogaiah S, Ito S, Nagaraj AK, Tran LP. 2015. Improvement of growth, fruit weight and early blight disease protection of tomato plants by rhizosphere bacteria correlated with their beneficial traits and induced biosynthesis of antioxidant peroxidase and polyphenol oxidase. Plant Sci 231:62-73.

Bauernfeind A, Schneider I, Jungwirth R, Roller C. 1999. Discrimination of *Burkholderia multivorans* and *Burkholderia vietnamiensis* from *Burkholderia cepacia genomovars* I, III, and IV by PCR. J Clin Microbiol 37 (5):1335-9.

Behera BC, Yadav H, Singh SK, Sethi BK, Mishra RJ, Kumari S, Thatoi H. 2017. Alkaline phosphatase activity of a phosphate solubilizing *Alcaligenes faecalis*, isolated from mangrove soil. Biotechnol Res Innov 1:101-110.

Blanco-Vargas A, Rodríguez-Gacha LM, Sanchez-Castro N, Garzon-Jaramillo R, Pedroza-Camacho LD,

Poutou-Pinales RA, …, Pedroza-Rodríguez AM. 2020. Phosphate-solubilizing *Pseudomonas* sp., and *Serratia* sp., co-culture for *Allium ce*pa L. growth promotion. Heliyon 6:e05218.

Brenner DJ, Krieg NR, Staley JR. 2005. Bergey's Manual Systematic Bacteriology, New York (US): Springer. p. 52-150.

Campos CA, Suarez MG, Laborde J. 2020. Analyzing vegetation cover-induced organic matter mineralization dynamics in sandy soils from tropical dry coastal ecosystems. Catena 185:104264.

Cherchali A, Boukhelata N, Kaci Y, Abrous-Belbachir O, Djebbar R. 2019. Isolation and identification of a phosphate-solubilizing *Paenibacillus polymyxa* strain GOL 0202 from durum wheat (*Triticum durum* Desf.) rhizosphere and its effect on some seedlings morphophysiological parameters. Biocatal Agric Biotechnol 19:101087.

Chowdhury SK, Majumdar S, Mandal V. 2020. Application of *Bacillus* sp. LBF-01 in *Capsicum annuum* plant reduces the fungicide use against *Fusarium oxysporum*. Biocatal Agric Biotechnol 27:101714.

Cui W, He P, Munir S, He P , Li X , Li Y, …, He Y. 2019. Efficacy of plant growth promoting bacteria *Bacillus amyloliquefaciens* B9601-Y2 for biocontrol of southern corn leaf blight. Biol Control 139: 104080.

da Silva K, Cassetari AD, Lima AS, Brandt ED, Pinnock E, Vandamme P, Moreira FMD. 2012. Diazotrophic Burkholderia species isolated from the Amazon region exhibit phenotypical, functional and genetic diversity. Syst Appl Microbiol 35:253-62.

de Castilho CL, Longoni L, J Sampaio J, Lisboa BB, Vargas LK, Beneduzi A. 2020. The rhizosphere microbiome and growth-promoting rhizobacteria of the Brazilian juçara palm. Rhizosphere 15:100233.

De Smet B, Mayo M, Peeters C, Zlosnik JE, Spilker T, Hird TJ, …, Vandamme P. 2015. *Burkholderia stagn*alis sp. nov. and *Burkholderia territorii* sp. nov., two novel *Burkholderia cepacia* complex species from environmental and human sources. Int J Syst Evol Microbiol. 65(7): 2265-71.

Guleria S, Walia A, Chauhan A, Shirkot CK. 2016. Molecular characterization of alkaline protease of *Bacillus amyloliquefaciens* SP1 involved in biocontrol of *Fusarium oxysporum*. Int J Food Microbiol 232:134-43.

Haile D, Firew Mekbib F, Assefa F. 2016. Isolation of phosphate solubilizing bacteria from white lupin (*Lupinus albus* L.) rhizosphere soils collected from Gojam, Ethiopia. J Fertil Pestic 7(2):1000172.

Hillis DM, Bull JJ. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Biol 42(2):182-92.

Hossain MI, Mizan MFR, Ashrafudoulla M, Nahar S, Joo HJ, Jahid IK, …, Ha SD. 2020. Inhibitory effects of probiotic potential lactic acid bacteria isolated from kimchi against *Listeria monocytogenes* biofilm on lettuce, stainless-steel surfaces, and MBEC™ biofilm device. LWT-Food Sci Technol 118: 108864.

Jangir M, Pathak R, Sharma S, Sharma S. 2018. Biocontrol mechanisms of *Bacillus* sp., isolated from tomato rhizosphere, against *Fusarium oxysporum* f. sp. *lycopersici*. Biol Control 123:60-70.

Joe MM, Deivaraj S, Abitha Benson A, Henry AJ, Narendrakumar G. 2018. Soil extract calcium phosphate media for screening of phosphate-solubilizing bacteria. Agric Nat Resour 52:305-308.

Karthika S, Midhun SJ, Jisha MS. 2020. A potential antifungal and growth-promoting bacterium *Bacillus* sp. KTMA4 from tomato rhizosphere. Microb Pathog 142:104049.

Khedhera SB, Mejdoub-Trabelsib B, Tounsia S. 2021. Biological potential of *Bacillus subtilis* V26 for the control of *Fusarium* wilt and tuber dry rot on potato caused by *Fusarium* species and the promotion of plant growth. Biol Control 152:104444.

Kumar H, Dubey RC, Maheshwari DK. 2017. Seed-coating fenugreek with *Burkholderia* rhizobacteria enhances yield in field trials and can combat *Fusarium* wilt. Rhizosphere 3:92-9.

Lau ET, Tani A, Khew CY, Chua YQ, Hwang SS. 2020. Plant growth-promoting bacteria as potential bio-inoculants and biocontrol agents to promote black pepper plant cultivation. Microbiol Res 240:126549.

LiPuma JJ, Dulaney BJ, McMenamin JD, Whitby PW, Stull TL, Coenye T, Vandamme P. 1999. Development of rRNA-based PCR assays for identification of *Burkholderia cepacia* complex isolates recovered from cystic fibrosis patient. J Clin Microbiol 37(10):3167-70.

Liu Z, Li YC, Zhang S, Fu Y, Fan X, Patel JS, Zhang M. 2015. Characterization of phosphate-solubilizing bacteria isolated from calcareous soils. Appl Soil Ecol 96:217-24.

Ludueña SLM, Anzuay MS, Angelini JG, McIntosh M, Becker A, Rupp O, …, Taurian T. 2018. Strain *Serratia* sp. S119: A potential biofertilizer for peanut and maize and a model bacterium to study phosphate solubilization mechanisms. Appl Soil Ecol 126:107-12.

Mahdavi P, Bergmeier E. 2016. Plant functional traits and diversity in sand dune ecosystems across different biogeographic regions. Acta Oecol 74:37-45.

Mardad IA, Serrano A, Soukri A. 2013. Solubilization of inorganic phosphate and production of organic acids by bacteria isolated from a Moroccan mineral phosphate deposit. Afr J Microbiol Res 7(8):626-35.

Nautiyal CS. 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265-70.

Panda B, Rahman H, Panda J. 2016. Phosphate solubilizing bacteria from the acidic soils of Eastern Himalayan region and their antagonistic effect on fungal pathogens. Rhizosphere 2:62-71.

Pastore G, Kernche S, Spohn M. 2020. Microbial solubilization of silicon and phosphorus from bedrock in relation to the abundance of phosphorus-solubilizing bacteria in temperate forest soils. Soil Biol Biochem 151:108050.

Paul D, Sinha SN. 2013. Isolation of phosphate solubilizing bacteria and total heterotrophic bacteria from river water and study of phosphatase activity of phosphate solubilizing bacteria. Adv Appl Sci Res 4:409-12.

Rasool A, Mir MI, Zulfajri M, Hanafiah MM, Unnisa SA, Mahboob M. 2021. Plant growth promoting and antifungal asset of indigenous rhizobacteria secluded from saffron (*Crocus sativus* L.) rhizosphere. Microb Pathog 150:104734.

Rasul M, Yasmina S, Zubaira M, Mahreena N, Yousaf S, Arifa M, …, Mirza MS. 2019. Phosphate solubilizers as antagonists for bacterial leaf blight with improved rice growth in phosphorus deficit soil. Biol Control 136:103997.

Rasul M, Yasmin S, Yahya M, Breitkreuz C, Tarkka M, Reitz T. 2021. The wheat growth-promoting traits of *Ochrobactrum* and *Pantoea* species, responsible for solubilization of different P sources, are ensured by genes encoding enzymes of multiple P-releasing pathways. Microbiol Res 246:126703.

Royse DJ, Ries SM. 1978. The influence of fungi isolated from peach twigs on the pathogenicity of *Cytospora cincta*. Phytopathology 68(4):603-7.

Sahandi MA, Mehrafarin A, Badi HN, Khalighi-Sigaroodi F, Sharifi M. 2019. Improving growth, phytochemical, and antioxidant characteristics of peppermint by phosphate-solubilizing bacteria along with reducing phosphorus fertilizer use. Ind Crops and Prod 141:111777.

Santos-Torres M, Romero-Perdomo F, Mendoza-Labrador J, Guti´errez AY, Vargas C, Castro-Rincon E, …, Estrada-Bonilla GA. 2021. Genomic and phenotypic analysis of rock phosphate-solubilizing rhizobacteria. Rhizosphere 17:100290.

Schlaberg R, Simmon KE, Fisher MA. 2012. A systematic approach for discovering novel, clinically relevant bacteria. Emerg Infect Dis 18 (3):422-30.

Simonetti E, Roberts IN, Montecchia MS, Flavio H. Gutierrez-Boem FH, Gomez FM, …, Ruiz JA. 2018. A novel *Burkholderia ambifaria* strain able to degrade the mycotoxin fusaric acid and to inhibit *Fusarium* spp. growth. Microbiol Res 206:50-9.

Srinivas C, Devi DM, Murthy KN, Mohan CD, Lakshmeesha TR, Singh BP, …, Srivastava RK. 2019. *Fusarium oxysporum* f. sp. *lycopersici* causal agent of vascular wilt disease of tomato: Biology to diversity-A review. Saudi J Biol Sci 26:1315-24.

Tagele SB, Kim SW, Lee HG, Kim HS, Lee YS. 2018. Effectiveness of multi-trait *Burkholderia contaminans* KNU17BI1 in growth promotion and management of banded leaf and sheath blight in maize seedling. Microbiol Res 214:8-18.

Tapia-García EY, Hernández-Trejo V, Guevara-Luna J, Rojas-Rojas FU, Arroyo-Herrera I, Meza-Radilla G, …, Santos PE. 2020. Plant growth-promoting bacteria isolated from wild legume nodules and nodules of *Phaseolus vulgaris* L. trap plants in central and southern Mexico. Microbiol Res 239:126522.

Teymouri M, Akhtari J, Karkhane M, Marzban A. 2016. Assessment of phosphate solubilization activity of rhizobacteria in a mangrove forest. Biocatal Agric Biotechnol 5:168-72.

Valetti L, Liliana IL, Fabra A. 2018. Growth promotion of rapeseed (*Brassica napus*) associated with the inoculation of phosphate solubilizing bacteria. Appl Soil Ecol 132:1-10.

Vanlaere E, Lipuma JJ, Baldwin A, Henry D, De Brandt E, Mahenthiralingam E, …, Vandamme P. 2008. *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica* sp. nov., novel species within the *Burkholderia cepacia* complex. Int J Syst Evol Microbiol 58 (PT 7):1580-90.

Viallard V, Poirier I, Cournoyer B, Haurat J, Wiebkin S, Ophel-Keller K, Balandreau J. 1998. *Burkholderia graminis* sp. nov. a rhizospheric *Burkholderia* species, and reassessment of [*Pseudomonas*] *phenazinium*, [*Pseudomonas*] *pyrrocinia* and [*Pseudomonas*] *glathei* as Burkholderia. Int J Syst Bacteriol 48 (PT 2):549-63.

Widane KA, Retnaningrum E. 2018. Kemampuan bakteri pelarut fosfat dan antipatogen *Fusarium oxysporum* (layu fusarium) isolat rhizosfer *Fimbristylis cymosa* R. Br. dari gumuk pasir Pantai Parangtritis Yogyakarta. [The capability of phosphate solubilizing bacteria and antipathogen *Fusarium oxysporum* isolated from the rhizosphere of *Fimbristylis cymosa* R.Br. obtained in sand dunes habitat in Parangtritis Coastal Area of Yogyakarta]. Final Research Project of Undergraduate Student. Yogyakarta (ID): Universitas Gadjah Mada.

Yang F, Sui L, Tang C, Li J, Cheng K, Xue Q. 2021. Sustainable advances on phosphorus utilization in soil via addition of biochar and humic substances. Sci Total Environ 768:145106.

Yarza P, Sproer C, Swiderski J, Mrotzek N, Spring S, Tindall BJ, …, Rossello-Mora R. 2013. Sequencing orphan species initiative (SOS): Filling the gaps in the 16S rRNA gene sequence database for all species with validly published names. Syst Appl Microbiol 36(1):69-73.

Zhang X, Yingying Zhou Y, Yan Li Y, Fu X, Wang Q. 2017. Screening and characterization of endophytic *Bacillus* for biocontrol of grapevine downy mildew. Crop Prot 96:173-9.

Zhao K, Penttinen P, Zhang X, Ao X, Liu M, Yu X, Chen Q. 2014. Maize rhizosphere in Sichuan, China, hosts plant growth-promoting *Burkholderia cepacia* with phosphate solubilizing and antifungal abilities. Microbiol Res169:76-82.

MOLD DIVERSITY OF WREATHED HORNBILL (*Rhyticeros undulatus*) NEST IN MOUNT UNGARAN

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ABSTRACT

Wreathed Hornbill (*Rhyticeros undulates*) is known to build nests in three cavities where they managed to live and breed. This edifice is predicted to contain various molds needed to maintain micro-environmental steadiness. This study was aimed to identify molds diversity in the Wreathed Hornbill's nest, using samples collected from empty structure with no bird activity. The samples were obtained from the Kalisidi and Nglimut observation stations on two occasions, i.e., in 2016 and 2017. Furthermore, the samples comprised cover soil, wood and inner material, which were collected aseptically and placed in sterile ziplock plastic bags. These samples were then diluted in sterilized distilled water to attain 10^{-3} mg/mL, and subsequently inoculated on Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Czapek Dox Agar (CDA). The inoculants were incubated at 37 °C, followed by the observation of mold colony after the 11th day. The results identified seven and nine species of molds in the Kalisidi and Nglimut observation stations, respectively. The most abundant species was *Penicillium* sp. which was found in composted nest materials for the whole observation periods.

Keywords: mold diversity, Mount Ungaran, nest, *Rhyticeros undulates*, Wreathed Hornbill

INTRODUCTION

Wreathed Hornbill or *Rhyticeros undulauts* Shaw 1811 is a protected bird, which is categorized into Apendix II, according to the *Convention on International Trade of Endangered Spesies of Wild Fauna and Flora* (CITES). Appendix II lists "all species which although not necessarily now threatened with extinction may become so, unless trade in specimens of such species is subject to strict regulation". This is an indication that the species is tradable under certain conditions, e.g., for scientific research purposes (Rahayuningsih & Kartijono 2013). In addition, breeding and nesting occur during fruiting season. The Wreathed Hornbill selects nest location based on the availability of fruiting trees and a conducive environment (Rahman *et al.* 2019). The nest selection process is followed by the nest building process, which involves the use of existing cavities from other birds and cracked branches (Rahayuningsih *et al. 2017)*. Generally, the nest selection and building process are initiated in dry season, characterized by low humidity, which is suitable for breeding and protecting the eggs from parasites. (Supa-Amornkul *et al.* 2011). However, the *R. undulatus* in Khao Yai, Thailand is capable of completing the breeding process before the heavy rains.

The created nests provide a suitable physical environment, alongside microorganisms needed for the development of eggs and chicks (Rahayuningsih *et al.* 2017; Utoyo *et al.* 2017). In addition, the strength of the physical structure is ensured by building the inner and outer parts of the nest using different soil sources, while the outer cover comprises high mircroorganism diversity, including mold. There have been minimal studies on mold diversity in *R. undulatus* nests present on Mount Ungaran, although Supa-Amornkul *et al.* (2011) reported the importance of micro-fungus in breaking down wood and organic materials. This phenomenon is exploited in nest cavity expansion; hence the study aimed to determine the diversity of molds in *R. undulatus* nests on Mount Ungaran.

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MATERIALS AND METHODS

This research was an observational exploratory study on the diversity of mold present in the nest of *R. undulatus*. The identification process was conducted in the Microbiology Laboratory, Department of Biology, Universitas Negeri Semarang (UNNES). Samples were obtained from the Kalisidi observation station located in the Kalisidi Village, West Ungaran District, Semarang, and Nglimut observation station located in Nglimut Village, Gonoharjo District, Kendal District, Central Java. The first observation period was conducted in 2016, and the last observation period was conducted in 2017. Samples were taken at each observation period.

Nest Sampling

The nest sampling process was performed at the end of the nesting season, marked by the absence of female and juvenile birds. Samples were collected aseptically, using sterile pinset and spatula, and then placed in ziplock plastic bags. The collected samples included nest cover, internal wood and nest materials of each nest in the two observation periods. All of the prepared samples were then stored in a freezer at -20 °C, prior to further analysis. Three samples were collected at each observation period. Thus, our study collected six samples altogether for the two observation period.

Each of the six samples were mashed up aseptically, followed by taking 10 g of each sample and dissolving the 10 g of each sample by using 100 mL of distilled water. This mixture was then homogenized and kept as a sample solution, from which 10% was diluted to attain 10^{-3}. Subsequently, the inoculation step was performed by spreading 1 mL of the sample solution onto culture media, i.e., Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Czapek Dox Agar (CDA). The isolates were then incubated at room temperature for 3-7 days in an incubator. A microscope was used for identification purposes, followed by the observation of wide mold colony growth.

Sterilization and Medium Preparation

The culture media used were Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Czapek Dox Agar (CDA). Each of the culture media was measured for 250 mL and dissolved with distilled water. As much as 0.05 g of chloramphenicol was added into the culture media as antibacterial agent, followed by sterilization using an autoclave at 121 °C and 2 atm, for 15 min. Subsequently, the culture media were poured into different Petri dishes under aseptic conditions, stored inside the Biological Safety Cabinet (BSC), and allowed to solidify, before being placed in a media cooler.

Calculating and Identification of Mold Colony

The grown mold colonies were stained using lactophenol, prior to observation with a microscope at magnifications of 100x - 1,000x. Subsequently, identification was carried out based on the guidelines of mold morphological structure developed by Samson *et al.* (2019) and Robinson (2011) to determine the genus and species of the samples.

RESULTS AND DISCUSSION

Environmental conditions of the Kalisidi and Nglimut observation stations are presented in Table 1.

Table 1 Enviromental condition of the observation stations

Enviromental conditions	Kalisidi station	Nglimut station
Light intensity (C)	613-1,740	656-1,480
Soil pH	5.2-6.2	3.7-4.5
Soil humidity (%)	25-55	70-90
Air humidity (%)	71-75	75
Air temperature °C	29.4-29.9	28.9
Tree type	Dead tree (unidentified)	*Weinmannia fraxenia* (nuts)

Despite the similarities, there was a variation in light intensity, with Nglimut station amounting 656 C to 1,480 C, due to the presence of tighter and thicker canopies. High and low light intensities impacted the air temperature around the nest, which was relatively higher in Kalisidi station. Kalisidi station featured higher soil pH and lesser soil humidity (Table 1).

After reaching a diameter of 1.5 cm, the mold colonies were transferred into the subsequent culture medium, followed by staining and observation by a microscope for identification purpose. Mold colonies having a diameter less than 1 cm were re-incubated to ensure further growth for easier identification. Table 2 shows the relatively higher density of samples obtained in 2017, compared to those obtained in 2016.

Within the incubated conditions, the MEA culture medium was colonized by various molds, where four and seven species were observed in samples obtained in 2016 and 2017, respectively. On the other hand, two and seven mold species grew in the PDA culture medium, while two and four mold species were recognized in CDA culture medium.

Aspergillus was successfully identified as the only genera present in all medium, across both sampling years. Furthermore, *Aspergillus niger* occurred less frequently than *A. terreus*, despite the fact that *A. niger* is commonly found in general environment. Meanwhile, *Acremonium* sp. and *P. variabile* were both identified in the MEA medium of 2016, while *Rhizopus* sp. was only seen in the CDA medium of 2017. Based on the collection period, there was a possibility that the mold species abundance was affected by the growth medium applied (Mahadevan & Shanmugasundaram 2018).

These mold species generally grow better in the MEA culture medium, made from wheat extract, maltosa, long chain carbohydrate and glycerol. The next culture medium preferred by mold to grow is PDA and followed by CDA. In addition, the composition of MEA provides sugar and nutrients as a source of energy for molds and yeast (Aziz *et al.* 2018; Cvetkovic & Markov 2002). The pepton content in MEA functions as a nitrogen source, presents in higher amounts compared to other media. Pepton is important for amino acid synthesis, which is required in the production of various functional protein, cell structure and hyphae conformation (Wang *et al.* 2010) and is responsible for the relatively higher amount of essential amino acid in MEA, including triptophan and tyrosin. These are important components in the conformation of metabolic protein, and also during cell communication.

PDA media includes the semi synthetic variety, resulting from the natural and synthetic material (*dextrose* and agar) components. In addition, complex carbohydrate molecules have been identified as the main source of carbon in potato. Carbon is the base material for PDA production, needed for the growth of molds and yeast. PDA is used because of the vitamin and mineral contents required to support fungi growth. Also, the dextrose component in PDA provides additional sugar as an energy source.

Table 2 Types of mold grown in the three culture media

Species	2016			2017		
	PDA	MEA	CDA	PDA	MEA	CDA
Aspergillus sp.	√	√	√	√	√	√
Aspergillus niger	-	√	-	-	-	-
Aspergillus terreus	√	-	-	√	√	-
Acremonium sp.	-	√	-	-	-	-
Curvularia sp.	-	-	-	√	√	-
Fusarium sp.	-	-	-	-	√	√
Geotrichum sp.	-	-	-	√	√	-
Neosartorya fischeri	-	-	-	√	√	-
Penicillium sp.	-	-	√	√	√	-
Penicillium variabile	-	√	-	-	-	-
Rhizopus sp.	-	-	-	-	-	√
Trichoderma sp.	-	-	-	√	-	√
Total spesies	2	4	2	7	7	4

Notes: PDA = Potato Dextrose Agar; MEA = Malt Extract Agar; CDA = Czapek Dox Agar; 2016 and 2017 = sampling years.

On the other hand, CDA medium consists of various nutrient molecules, of which sucrose has been identified as the main energy source, while nitrogen is obtained from the natrium nitrate component. Furthermore, other constituents, including dipotassium phosphate is known to serve as a buffer solution, while magnesium sulfate and iron sulfate are essential ions. However, both MEA and PDA used in this study are manufactured medium, ready to use, while CDA was created using a formula according to the manufacture's protocol (HiMedia Laboratories, Mumbai), hence the tendency for unclear and unstandardized composition accuracies and capabilities.

The molds obtained in 2016 at the Kalisidi station were successfully identified, and three species were observed in the first collection (P1), with two in the second collection (P2). Two species were accumulated in P1, and four species in P2 for samples collected in 2017. However, the Nglimut station portrayed a relatively higher diversity (Table 3).

Aspergillus sp. was the only species present five times in the *R. undulatus* nest, i.e., two times in Kalisidi station and three times in Nglimut station, while *Penicillium* sp. were observed two times in each station. In addition, *A. terreus* was identified on three instances, i.e., one and two times for the respective stations. On the other hand, *Curvularia* sp., *Fusarium* sp., *Geotrichum* sp., *N. fischeri* and *Trichoderma* sp. were only identified twice in both stations, while *A. niger, Acremonium* sp., *P. variabile*, and *Rhizopus* sp. were rarely present (Table 4).

Table 3 Types of molds in the cover Wreathed Hornbill (*Rhyticeros* undulatus) obtained in the 2016 and 2017 sampling period

Species	Kalisidi station				Nglimut station			
	2016		2017		2016		2017	
	P1	P2	P1	P2	P1	P2	P1	P2
Aspergillus sp.	√	√	-	-	√	-	√	√
Aspergillus niger	-	-	-	-	√	-	-	-
Aspergillus terreus	-	√	-	-	-	-	√	√
Acremonium sp.	√	-	-	-	-	-	-	-
Curvularia sp.	-	-	-	-	-	-	√	√
Fusarium sp.	-	-	-	-	-	-	√	√
Geotrichum sp.	-	-	√	√	-	-	-	-
Neosartorya fischeri	-	-	-	√	-	-	-	√
Penicillium sp.	√	-	-	√	-	-	√	√
Penicillium variabile	-	-	-	-	√	-	-	-
Rhizopus sp.	-	-	-	-	-	-	-	√
Trichoderma sp.	-	-	√	√	-	-	-	-
Total spesies	3	2	2	4	3	-	5	7

Notes: P1 = first collection; P2 = second collection.

Table 4 Types of molds in *R. undulatus* nest during sampling in 2017

Types of molds	Kalisidi station						Nglimut station					
	P1			P2			P1			P2		
	CN	WM	CM	CN	WM	CM	CN	WM	CM	CN	WM	CM
Aspergillus sp.	-	-	-	-	-	-	√	√	√	√	√	√
Aspergillus niger	-	-	-	-	-	-	√	-	-	√	-	-
Aspergillus terreus	-	-	-	-	-	-	-	-	√	-	-	√
Acremonium sp.	-	-	-	-	-	-	-	-	√	-	-	-
Curvularia sp.	-	-	-	-	-	-	-	-	√	-	-	√
Fusarium sp.	-	-	-	-	-	-	√	√	-	√	√	√
Geotrichum sp.	√	-	-	-	-	√	-	-	-	-	-	-
Neosartorya fischeri	-	-	-	-	√	√	-	-	-	-	-	√
Penicillium sp.	-	-	-	-	-	√	√	√	√	√	√	√
Penicillium variabile	-	-	-	-	√	-	-	-	-	-	-	-
Rhizopus sp.	-	-	-	-	-	-	-	-	-	√	-	-
Trichoderma sp.	-	√	-	√	√	-	-	-	-	-	-	-
Total spesies	1	1	-	1	3	2	4	3	5	5	3	6

Notes: P1 = first collection; P2 = second collection; CN = cover nest; WM = wood material; CM = compost materi al.

Samples obtained from the nests were divided into three parts, i.e., cover, internal compost and wood material. The most abundant molds identified at the Kalisidi station included *Geotrichum* sp., *N. fischeri* and *Trichoderma* sp. The *Trichoderma* sp. Was observed in the first and second collection periods, while the wood material containing *N. fischeri* and *P. variabile* was observed in the second collection period. This result indicated that *N. fischeri* is the most widespread mold species.

The high diversity shown in Nglimut station included four mold species in the nest cover, with three in the wood material and four species from the compost. In addition, *Aspergilus* sp. and *Penicillium* sp. were identified as the most common molds in all parts, during both collection periods, while *A. niger* was only found in the nest cover, with *A. terreus* in the nest inner material for both collection periods .

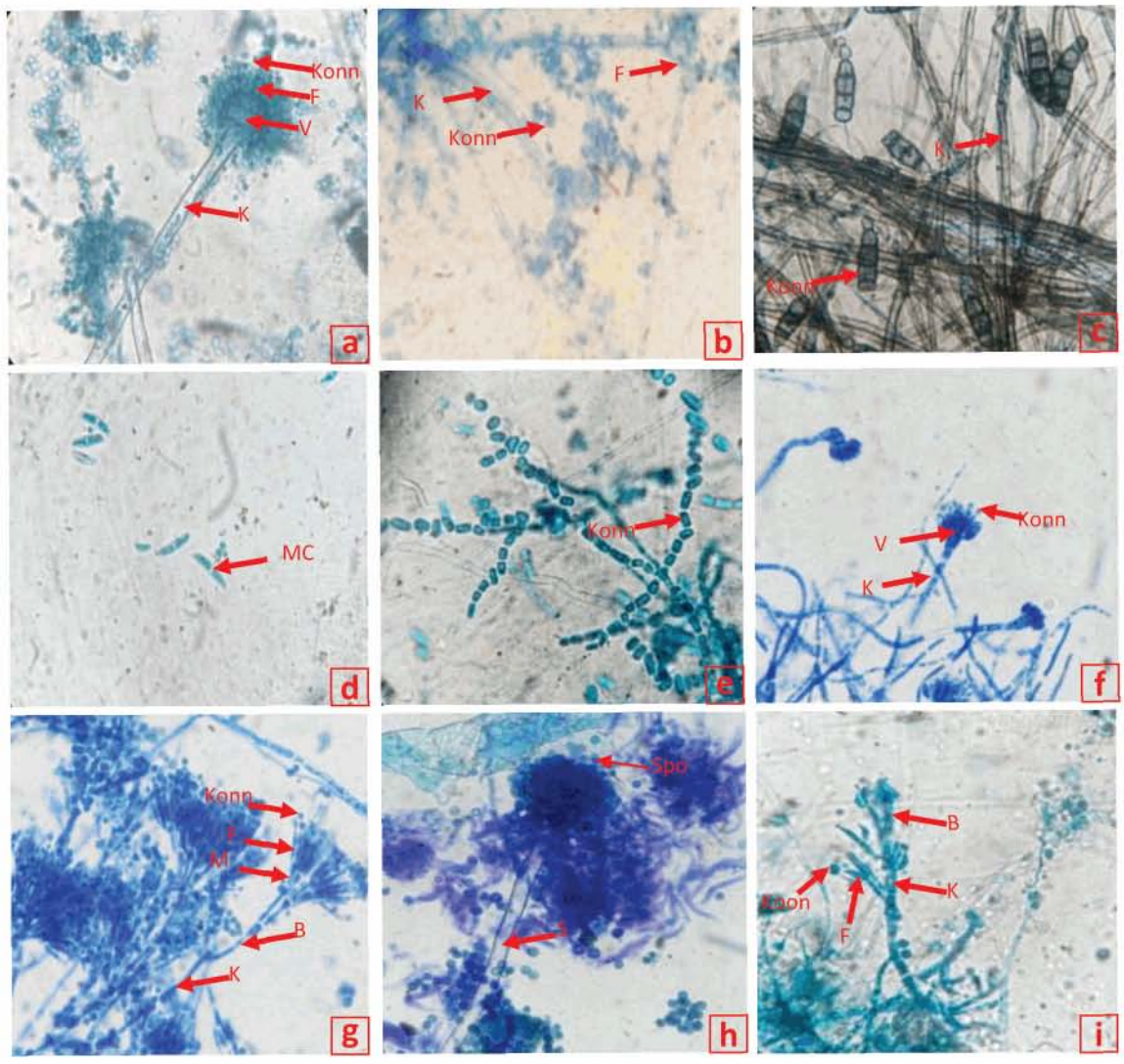

Figure 1 Molds types identified in *R. undulatus* nest
Notes: a = *Aspergillus* sp.; b = *Acremonium* sp.; c = *Curvularia* sp.; d = *Fusarium* sp.; e = *Geotrichum* sp.; f = *Neosartorya fischeri*; g = *Penicillium* sp.; h = *Rhizopus* sp.; i = *Trichoderma* sp. (B = Branch; F = Phialid; K = Conidiophore; Kon = Conidia; M = Metula; MC = Microconidia; S = Sporangiophore; Spo = Spora; V = Vesicle). Microscope magnification: 1,000x.

The morphologycal identification process was based on two main characteristics, including: 1) the colony formation and color and 2) the morphological structure. Based on these two main characteristics, the molds were identified into genus and species (Table 5).

Table 5 Description of molds types identified in the nest of *R. undulatus*

Mold	Description
Aspergillus sp.	The fruit body consisted of *Aspergillus* formed conidiophores (non-septate), vesicles, metula, phialid, stolone (vegetative hyphae) and conidia. The identified *Aspergillus* sp. possessed radiate- and biseriate-conidial heads (sideways/ deviate), with phialid organs that grow in the metula, as seen in Figure 1.a. Hence, molds with similar criteria were designated as *Aspergillus* (Diba *et al.* 2007)
Acremonium sp.	The typical organs present included a cluster of aerial hyphae, conidiophores, phialids and ellipse extended conidia. Furthermore, the phialid grew directly on aerial hyphae, and was tapered in the form of a needle, while the conidiophores were single-celled, erected and unbranched condia (Fig. 1.b) (Samson *et al.* 1984). The microscopic size range of these components were 17.5-37.5 (-50.4) × 3.2-4 µm, and 6-8.5 (-9.3) × 2.1-2.8 (-4) µm, respectively (Gräfenhan *et al.* 2011; Hill *et al.* 1990).
Curvularia sp.	The conidiophores were branched, brown, and tightly arranged in groups. In addition, the conidia were elliptical with 3-4 bulkheads in each, with brownish white coloration, and comprising of 4-5 cells. The colonies were dark black in color and round, with cotton texture (Fig. 1.c). This was in accordance with the description by Kusai (2015) and Hosokawa (2003), except with the addition of velvet pigmentation. The conidiophores appeared singly or in groups, simple or branched, straight or crouched, with pale brown or young cones, while the conidia had 3-4 septa. The specimen was thin-walled, measuring 20-30 x 9-15 µm (Hosokawa *et al.* 2003; Kusai *et al.* 2016).
Fusarium sp.	The *Fusarium* genera was identified using the method by (Bashyal *et al.* 2016; Gräfenhan *et al.* 2011). The microconidia appeared as fusiform and ovoid form, with 0-1 septate, while the conidiophorous structure present was insulated. In addition, phialid and macroconidia were not seen under microscopic observations, although microconidia were recognized (Fig. 1.d).
Geotrichum sp.	Conidia were cylindrical, oval, and tubular (barrel) in shape, with green-blue coloration, and also a chain-like and clustered arrangement. In addition, the upper part of this mold was formed from broken fertile hyphae (Fig. 1.e), with conidia diameter of 3.7-4.8-12.5 (-13.8) x (1.7-) 2.4-5 µm, and no conidiophores. Also, the fertile hyphae present was branched off dichotomous and insulate.
Neosartorya fischeri	The morphological structure of *Neosartorya fischeri* was similar with *Aspergillus*, characterized by vesicles, phialid and conidia, with seemingly insulated hyphen, alongside blue densely arranged conidiophores and hyphae. In addition, the vesicles were slightly elongated in shape, with conidial columnar head, which was also uniseriate (direct phialid growth in vesicles) (Fig. 1.f). The colony was white in color, with cotton-like texture, and ± 0.2-2 cm in diameter. The conidia of *Neosartorya fischeri* was round in shape, half round and elliptical, with slightly coarse wall, at ± 2-3 µm diameter, while the conidiophores ranged from 300-500 µm, with characteristic smooth walls (Udagawa *et al.* 1996).
Penicillium sp.	The morphological structures possessed insulated vegetative hyphae, alongside conidiophores, branches, metula, phialid and conidia. The conidiophores were of the two-stage branched (biverticillate-asymmetrical) type, while the conidia appeared round (Fig. 1.g). In addition, the colonies were grayish and light green-old, with ± 0.2–2 cm diameter. Also, *Penicillium* is included as a Deuteromycota, characterized by fast growing colonies, which is green in appearance and sometimes white. The conidiophores had several branching pattern forms, including one to three-stages and more-stage branched (Visagie *et al.* 2014).
Rhizopus sp.	The morphological structure had sporangiofor, sporangium, featuring the release of spores (sporangiospor). In addition, the mold contains the non-septate stolone (vegetative hyphae), alongside the rhizoid, although only the columella covered by sporangium. The Sporangiofor stands tall, with a round shape (Fig. 1.h) (Hartanti *et al.* 2015).
Trichoderma sp.	Morphological structures are similar with *Trichoderma*, featuring vegetative hyphae (aerial hypha), conidiophores with side branches, slim and elongated phialids, and also round conidia, with white and dark green colonies (Fig. 1.i). According to Gusnawaty *et al.* (2014), *Trichoderma* sp. has branched conidiophores resembling pyramids, with more to the end, and the branching becomes shorter. Also, the conidia are smooth walled and semi-round to oval in shape. This species have green colonies that were initially white (Supa-Amornkul *et al.* 2011).

The nest cover collected in this study consisted of soil and wood. Molds identified from the nest cover were of various species, although the more abundant molds were observed in the organic material of the nest's inner part. This was possibly caused by composted organic materials, including feces, fermented fruit, e.g., *Ficus*, insects and decayed wood. Particularly, the *Ficus* fruit or fig (*Ficus carica*) contains 8.98% protein, 6.57% fat, 10.26% moisture content, 18.23% ash content, 20.31% crude fiber, 0.0395% calcium, 0.002% phosphor, 25.48 mg/100 g and 1.64 mg/100 g of vitamin C and E, respectively. In addition, fig also contains various minerals needed by *R. undulates,* including N, P, K, Ca, Mg and others (Mendoza-Castillo 2019). Aside from fruits, the fecal matter was high in N for mold protein synthesis, while the soil and wood were characterized by water, fat, carbohydrate and protein. This results were confirmed with the results of previous studies on the nest of *R. undulatus* containing 53.30 mg/mL of water, 39.02 mg/mL of fat, 35.03 mg/mL of carbohydrates, 5.82 mg/mL of ash and 20.12 mg/mL of protein. In addition, the humidity and the warm and dark conditions of the inner part of the nest form an appropriate and suitable environment for mold growth.

CONCLUSION

Mold species obtained from *R. undulatus* nests consisting of cover, composted material and wood material in Kalisidi and Nglimut stations during the sampling in 2016 comprised 6 mold species including *Aspergillus niger, Aspergillus terreus, Aspergillus* sp., *Penicillium* sp., *Penicillium variabile* and *Acremonium* sp. On the other hand, 9 mold species were reported during the sampling in 2017, including *Aspergillus terreus, Aspergillus* sp., *Curvularia* sp., *Fusarium* sp., *Penicillium* sp., *Rhizopus* sp., *Geotrichum* sp., *Trichoderma* sp., and *Neosartorya fischeri.*

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REFERENCES

Aziz NHA, Yousef NS, El-Hadded ME, El-Tayeb TS. 2018. Influence of nutritional and climatic conditions on mycelial growth of three oyster mushroom strains. Arab Univ J Agric Sci 26: 1165-73.

Bashyal BM, Aggarwal R, Sharma S, Gupta S, Rawat K, Singh D, Singh AK, Krishnan SG. 2016. Occurrence, identification and pathogenicity of *Fusarium* species associated with bakanae disease of basmati rice in India. Eur J Plant Pathol 144: 457-66.

Cvetkovic D, Markov S. 2002. Cultivation of tea fungus on malt extract medium. Acta Period Technol 33:117-24.

Diba K, Kordbacheh P, Mirhendi SH, Rezaie S, Mahmoudi M. 2007. Identification of *Aspergillus* species using morphological characteristics. Pakistan J Med Sci 23:867-72.

Gräfenhan T, Schroers HJ, Nirenberg HI, Seifert KA. 2011. An overview of the taxonomy, phylogeny, and typification of nectriaceous fungi in *Cosmospora, Acremonium, Fusarium, Stilbella*, and *Volutella*. Stud Mycol 68:79-113.

Hartanti AT, Rahayu G, Hidayat I. 2015. *Rhizopus* species from fresh tempeh collected from several regions in Indonesia. Hayati J Biosci 22:136-42.

Hill NS, Stringer WC, Rottinghaus GE, Belesky DP, Parrott WA, Pope DD, Agronomy D. 1990. Growth, morphological, and chemical component responses of tall fescue to *Acremonium coenophialum*. Crop Sci 30:156-61.

Hosokawa M, Tanaka C, Tsuda M. 2003. Conidium morphology of *Curvularia geniculat*a and allied species. Mycoscience 44:227-37.

Kusai NA, Mior MZA, Zulkifly S, Yusof MT, Mohd Zainudin NAI. 2016. Morphological and molecular characterization of *Curvularia* and related species associated with leaf spot disease of rice in Peninsular Malaysia. Rend Lincei 27:205-14.

Mahadevan K, Shanmugasundaram K 2018. Comparative effect of different culture media on mycelial growth performance of *Pleurotus sapidus*. J Pharmacogn Phytochem 7:874-8.

Rahayuningsih M, Kartijono NE. 2013. Profil habitat julang emas (*Aceros undulatus*) sebagai strategi konservasi di Gunung Ungaran, Jawa Tengah. Indonesia [The habitat profile of the golden crown (*Aceros undulatus*) as a conservation strategy in Mount Ungaran, Central Java. Indonesia]. J Conserv 2:14-22.

Rahayuningsih M, Kartijono NE, Retnoningsih A. 2017. Short communication: The nest characteristics of wreathed hornbill (*Rhyticeros undulatus*) in Mount Ungaran, Central Java, Indonesia. Biodiversitas, 18(3):1130-4.

Rahman F, Ismail A, Nurul-Huda MJ. 2019. Food items and foraging sites of the oriental pied-hornbill (*Anthracoceros albirostris*) during breeding season in sungai panjang, sabak bernam, Malaysia. Pertanika J Trop Agric Sci 42:251-9.

Robinson M. 2011. Pictorial atlas of soil and seed fungi: morphologies of cultured fungi and key to species (3rd ed.). Ref Rev 25(4):43-4.

Samson RA, Houbraken J, Thrane U, Frisvad JC. 2019. Food and indoor fungi. Utrecht (NT): Westerdijk Fungal Biodiversity Institute.

Supa-Amornkul S, Wiyakrutta S, Poonswad P. 2011. Wood decay fungi in hornbill nest cavities in Khao Yai National Park, Thailand. Raffles Bull Zool Supplement 24:95-113.

Udagawa SI, Tsubouchi H, Toyazaki N. 1996. Isolation and identification of *Neosartorya* species from house dust as hazardous indoor pollutants. Mycoscience 37:217-22.

Utoyo L, Marthy W, Noske RA, Surahmat F. 2017. Nesting cycle and nest tree characteristics of the Helmeted Hornbill *Rhinoplax vigil*, compared to the Wreathed Hornbill *Rhyticeros undulatus*, in Sumatran lowland rainforest. Kukila 20:12-22.

Visagie CM, Hirooka Y, Tanney JB, Whitfield E, Mwange K, Meijer M, …, Samson RA. 2014. *Aspergillus, Penicillium* and *Talaromyces* isolated from house dust samples collected around the world. Stud Mycol 78:63-139.

Wang H, Ma F, Cheng L. 2010. Metabolism of organic acids, nitrogen and amino acids in chlorotic leaves of "Honeycrisp" apple (*Malus domestica* Borkh) with excessive accumulation of carbohydrates. Planta 232:511-22.

GENETIC VARIABILITY OF ARROWROOT (*Maranta arundinacea* L.) IN YOGYAKARTA PROVINCE, INDONESIA BASED ON ISSR ANALYSIS

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ABSTRACT

The cultivation of arrowroot (*Maranta arundinacea* L.) in Indonesia, particularly in Yogyakarta Province has a great potential to be developed. This study aimed to determine genetic variability and analyze the intraspecific relations of arrowroot using Inter Simple Sequence Repeats (ISSR) markers to be used as basic information in considering characters selection of arrowroot cultivation in Yogyakarta. Exploratory survey method was conducted in Gunungkidul, Kulon Progo, Sleman, and Bantul Districts to collect cultivar accessions. Accessions were replanted in Sawitsari Research Station. DNA isolation from the leaves of 7-month-old accessions was carried out using CTAB buffer solution. The DNA fingerprinting analysis was carried out using the result of DNA amplification with 4 ISSR markers. The polymorphism data were then used for phenetic analysis using UPGMA algorithm and Baroni-Urbani Busser similarity coefficient to form a dendrogram. A total of 5 local cultivars were found, identified as 'Sili', 'Sembowo', 'Sugo', 'Kebo', and 'Teropong'. Each cultivar showed distinct rhizome morphological characteristics. The ISSR-PCR analysis resulted in high polymorphism with a 68.17% polymorphism mean. The mean of polymorphic band was 6.75. The dendrogram was developed based on the analyses and consisted of 4 clusters with 80% similarity index. Cluster A, B.I. and B.II.b consisted of 'Sili', 'Teropong', and 'Kebo' cultivars, respectively, while cluster B.II.a gathered 'Sugo' and 'Sembowo' cultivars.

Keywords: cluster analysis, intraspecific classification, ISSR markers, polymorphism

INTRODUCTION

Arrowroot cultivation in Indonesia has a great potential to be developed. Flour made from arrowroot rhizome has high economic and nutritional values (Madineni *et al.* 2012; Guilherme *et al.* 2017). Arrowroot flour contains high protein, even greater than that of cassava flour (Aprianita *et al.* 2014), low glycemic index value and high fiber content. Therefore, food products made from arrowroot flour are easier to digest compared with those made from other types of flour (Lestari *et al.* 2017). Food products made by arrowroot fluor is also recommended for people with digestive disorder and the elderdy (Silva *et al.* 2000; Heredia-Zárate & Vieira 2005; Mason 2009; Silveira *et al.* 2013) Furthermore, arrowroot flour has long been used traditionally by the household and by the food industries as a basic ingredient for baby food. Further research revealed that arrowroot flour is potential to be used as a thickening agent in various industries, such as cosmetics, pharmaceutical and food industries (Kitahara *et al.* 2007). In addition to these benefits, the widespread arrowroot population in Indonesia, especially in Yogyakarta Province, is the main reason for developing arrowroot cultivation (Djaafar *et al.* 2010; Masitoh 2014).

Information obtained from molecular analysis of a plant population can identify genotype differences among individuals as a selection step in plant breeding programs. An efficient character selection process by relying on the information about the genetic variation of a wild plant population with a suitable

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breeding method has succeeded in developing modern cultivars, which has increased the yield of various food crops since the mid-20th century. Molecular markers applied to reveal genetic variabilities can thus, provide promising information in agriculture as well as in protecting crop variabilities (Govindaraj *et al.* 2015; Perez-de-Castro *et al.* 2012; Faraldo *et al.* 2003).

Inter Simple Sequence Repeat (ISSR) is one of molecular markers commonly used in the study of genetic diversity, phylogeny, gene detection, genome mapping and evolutionary biology in various types of plants (Reddy *et al.* 2002). The combination of using ISSR in PCR has been done in genetic fingerprinting (Blair *et al.* 1999), cultivar identification (Wang *et al.* 2009), phylogenetic analysis (Gupta *et al.* 2008) and assessment of hybridization (Wolfe *et al.* 1998). The ISSR marker uses a 16-25 bp single primer that is specific to target identical regions among microsatellites. Primers composed of eight repeating dinucleotide units, six repeating trinucleotide units or several repeating tetra- or pentanucleotide units with or without nucleotide anchorage system that targets the microsatellite region (Zietjiewicz *et al.* 1994).

Various types of molecular markers were used to reveal the genotype diversity of arrowroot, especially between the local varieties and cultivars. Two of the molecular markers are RAPD and ISSR markers (Pinto 2015; Asha *et al.* 2015). This study aimed to determine genetic variability and analyze the intraspecific relationship of arrowroot population in Yogyakarta Province, Indonesia by using the ISSR markers. The expected results were the diversity of arrowroot germplasm in Indonesia which can be used to strengthen the phenotypic characters variations that have been used in arrowroot cultivation and breeding.

MATERIALS AND METHODS

Plant Materials

Sample collection was carried out during the dry season, from August to September 2019 in 18 subdistricts in Sleman, Gunungkidul, Kulon Progo, and Bantul Districts in Yogyakarta Province using the exploratory survey method. The whole arrowroot rhizome was taken from each accession and then replanted in the Sawitsari Research Center Plantation, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta. Species identification was carried out using the key of determination from van Steenis (1975), Woodson and Schery (1980), Wu and Kennedy (2000), Hammel *et al.* (2014), Lim (2016). Meanwhile, the cultivar identification was carried out through interviews with arrowroot farmers. Fresh leaf from each 7-month-old accession was used for DNA extraction.

DNA Isolation

Genomic DNA from the finely ground leaves (0.5 g) was extracted using the modified CTAB method (Deswina *et al.* 2019). Extraction buffer consisted of 50 mM Tris-HCl (pH 8), 0.7 M NaCl, 0.1% β-mercaptoethanol, 10 mM EDTA, 0.1% cetyltriethylammonium bromide (CTAB). The DNA quality was checked in an agarose gel through the electrophoresis and then quantified by measuring the OD at 260 nm and 280 nm through the spectrophotometry. Purity of DNA sample was calculated from OD at a 260/280 ratio.

ISSR Analysis

In this recent study, a total of 4 ISSR primers were used for analysis. The primers are UBC 811, UBC 827, UBC 818 and UBC 825 (Table 1).

Table 1 ISSR markers details

Markers	Sequence (5' - 3')	Annealing temperature (°C)	Size range (bp)
UBC 811	$(GA)_8C$	53	300-2,000 (Asha *et al.* 2015; Kambale *et al.* 2018)
UBC 827	$(AC)_8G$	52	300-1,600 (Asha *et al.* 2015; Kambale *et al.* 2018)
UBC 818	$(CA)_8G$	45	350-1,600 (Asha *et al.* 2015)
UBC 825	$(AC)_8T$	52	400-2,000 (Asha *et al.* 2015; Kumar *et al.* 2010)

The extracted DNA was amplified in a Thermal Cycler (Bio-Rad). The reaction mixture consisted of 8.5 µL ddH_2O, 2.0 µL of each primer, 12.5 µL MyTaq polymerase and 2 µL DNA sample. The total reaction volume was made up to 25 µL using sterile distilled water. PCR was carried out in a master cycler gradient with a certain temperature setting (Table 2).

Table 2 PCR amplification setting (Asha *et al.* 2015)

Steps	Temperature (°C)	Time (minutes)	Cycles
Pre-denaturation	94	4	1
Denaturation	94	1	
Annealing	45-55	0.5	40
Extention	72	1	
Post Extention	72	4	1
Hold	4	∞	1

The annealing temperature was standardized for each primer. The results of amplification were separated by gel electrophoresis in 2% agarose gel with 1X TBE buffer and were imaged using gel documentation with observation through UV vis transilluminator.

Data Analysis

The analysis was carried out only with the unambiguously and reproducibly amplified ISSR bands. Those DNA bands were scored as present (1) or absent (0). Smeared and weak bands were excluded. The resulting binary data matrix was analyzed using MVSP (MultiVariate Statistical Program) version 2.0. The analysis aimed to examine genetic relationship among accessions by estimating the similarity index using the Baroni-Urbani Busser similarity coefficient. Cluster analyses were carried out on the same similarity coefficient with the Unweighted Pair-Group Method of Average (UPGMA) (Rohlf 2000).

RESULTS AND DISCUSSION

Plant Collection and Identification

Identification of local cultivars succeeded in revealing the existence of 5 cultivars, namely 'Sembowo', 'Sili', 'Sugo', 'Kebo', and 'Teropong'. The 'Sili' and 'Sembowo' cultivars were found to be widely distributed in 4 districts (Table 3).

DNA Extraction

DNA extraction using the CTAB method was carried out on the accessions 'Sembowo' (represented by SENG1), 'Sili' (represented by SIPE3), 'Sugo' (represented by SUBR1), 'Teropong' (represented by TENG1) and 'Kebo' (represented by KEKE1). The quantity of isolated DNA was tested by using spectrophotometry and showed a fairly high level of purity in four of the five accessions. KEKE1 accessions were known to be contaminated, but based on the results of the electrophoresis tests, this DNA sample could still be used. Results of DNA concentrations varied. Each accession had a good DNA concentration and met the standards. The results of the spectrophotometric test are shown in Table 4.

Table 3 Sampling collection

No	Sampling location			Accessions type	Accessions code
	District	Subdistrict	Village, Subvillage		
1	Bantul	Sedayu	Argodadi, Brongkol	'Sili' (besar)	SIBB1
2	Bantul	Sedayu	Argodadi, Brongkol	'Sili' (kecil)	SIKB1
3	Bantul	Sedayu	Argodadi, Brongkol	'Sili' (bengkok)	SIEB1
4	Bantul	Sedayu	Argodadi, Brongkol	'Sugo'	SUBR1
5	Sleman	Pajangan	Triwidadi, Ngincep	'Sembowo'	SENG1
6	Sleman	Pajangan	Triwidadi, Ngincep	'Sili'	SING1
7	Bantul	Pajangan	Triwidadi, Ngincep	'Teropong'	TENG1
8	Bantul	Pleret	Wonolelo, Kedungrejo	'Kebo'	KEKE1
9	Gunungkidul	Wonosari	Gari, Gondangrejo	'Sembowo'	SEGO2
10	Kulon Progo	Pengasih	Pengasih, Pengasih	'Sembowo'	SEPE3
11	Kulon Progo	Pengasih	Pengasih, Pengasih	'Sili'	SIPE3
12	Kulon Progo	Pengasih	Sendangsari, Gegunung	'Sembowo'	SEGE3
13	Kulon Progo	Pengasih	Sendangsari, Gegunung	'Sili'	SIGE3
14	Sleman	Moyudan	Sumberagung, Kaliduren III	'Sembowo'	SEKD4
15	Sleman	Prambanan	Sumberharjo, Sengir	'Sembowo'	SESE4

Table 4 Spectrophotometry results

Accession Code	OD 260	OD 280	OD at 260/280 ratio	Concentration (ng/μL)
SENG1	7.03	3.67	1.929	365.52
SIPE3	2.54	1.35	1.898	127.32
SUBR1	4.62	2.43	1.917	231.11
KEKE1	5.57	9.95	1.371	678.87
TENG1	3.82	1.95	1.970	191.00

Each sample of isolated DNA concentrations showed various values ranging from 127.32 ng/μL to 678.87 ng/μL. The results indicated that isolated DNA from 'Sili' leaves produced the lowest concentration. However, the results also showed a fairly high absorbance ratio value of 1.898 at OD ratio 260/280. The OD value shows the purity of the DNA sample. There was only DNA sample of the local cultivar 'Kebo' which had a very low absorbance ratio value of 1.371 at OD ratio 260/280. The quantity of DNA concentration and quite low purity could be caused by polyphenol contamination. The quality of the DNA sample from 'Kebo' cultivar was still good and clearly showed the DNA bands.

The absorbance purity ratio of 260/280 is a very important measurement for estimating polyphenol contamination in the extracted DNA samples. Ratio of A260/A280 below 1.8 shows a poor DNA extraction result and is not recommend for molecular analysis (Sambrook & Russell 2001). A high purity ratio of A260/A280 also indicates the purity of DNA from RNA contamination (Koetsier & Cantor 2019). The use of high enough mercaptoethanol can replace the role of RNase in degrading and removing RNA from DNA samples, even though this reducing agent is toxic and pungent (Mommaerts *et al.* 2015).

Isolation of plant DNA using the CTAB protocol has many advantages and is proven to produce more sample volumes with high DNA concentrations. CTAB buffer solutions generally play a role in damaging cell structures, from cell walls to cell membranes and also the nuclear membrane. This is solely done to release the genetic material from the nucleus (Amani *et al.* 2011). CTAB buffer solution contains 2-β-mercaptoethanol which has been shown to completely remove polyphenol components in cells (Horne *et al.* 2004; Li *et al.* 2007). In this study, a buffer solution with a concentration of 0.1% mercaptoethanol was used and proved to be optimal in removing polyphenol contamination. Meanwhile, another study revealed that the use of 0.3% mercaptoethanol could improve the quality of DNA pellets from precipitation (Suman *et al.* 1999).

Analysis of ISSR-PCR

Molecular analysis used four ISSR markers, namely UBC 811, UBC 818, UBC 825 and UBC 827. PCR temperature optimization for these four markers resulted in different optimum annealing temperatures between UBC 827 & UBC 825 and UBC 818 & UBC 811. UBC 827 and UBC 825 markers require an annealing temperature of 50 °C, while the other two markers require an annealing temperature of 55 °C.

Electrophoresis of PCR-ISSR results showed variations in the number of DNA bands, polymorphic bands and different monomorphic bands among ISSR markers (Table 5).

Table 5 Number of DNA bands and polymorphism (%) in each molecular marker

ISSR markers	Sequences (5'- 3')	Total DNA Bands	Total Polymorphic DNA Bands	Polymorphisms (%)
UBC 811	$(GA)_8C$	11	8	72.7
UBC 818	$(AC)_8G$	8	4	50
UBC 825	$(CA)_8G$	10	8	80
UBC 827	$(AC)_8T$	10	7	70
	Average	9.75	6.75	68.17

Visualization of the electrophoresis results using the Gel-Doc Transilluminator showed variations among these molecular markers (Fig. 1). The average polymorphism appeared was 68.17% with the mean number of polymorphic bands of 6.75 and the average number of DNA bands of 9.75. UBC 811 primer produced the highest number of DNA bands, while UBC 811 and UBC 825 primers produced the highest number of polymorphic DNA bands with eight bands. Amplification using UBC 825 primer resulted in the highest polymorphism at 80%.

Electrophoresis using a DNA ladder with a size of 100 bp and a buffer solution of 2% TBE 1X showed that the size of the DNA bands in each primer was different, but there were several monomorphic bands with the same size between one marker and another. The DNA bands were 750 bp, 600 bp, and 450 bp. Overall, the size of DNA bands appeared to be ranging from 180 bp to 1,000 bp. Meanwhile, the polymorphic bands showed by each marker were diverse. In UBC 811, polymorphic DNA bands were found to have sizes of 200-300 bp, 300-400 bp and 800 bp. UBC 818 primer amplification resulted in the lowest polymorphism value, with only 50% polymorphism showed by the DNA bands 250 bp, 650 bp and 500-600 bp.

ISSR marker is an ideal genetic marker for various studies, most notably on genetic variation (Shafiei-Astani *et al.* 2015) and DNA fingerprinting (Shen *et al.* 2006). As a good genetic marker, ISSR produces high genetic variability and shows multilocus data due to the use of the highly variable microsatellite sequences that are ubiquitously distributed across the genome (Anne 2006; Tautz & Renz 1984; Wolff *et al.* 1995). The addition of one or more nucleotide anchors in ISSR marker to target the end of the microsatellite region can prevent primer dimerization (Bani *et al.* 2017). Based on comparison with other molecular markers, the ISSR marker has higher reproducibility compared to the Random Amplified Polymorphic DNA (RAPD). The ISSR marker is more time and money efficient compared to the Amplified Fragments Length Polymorphism (AFLP) (Phong *et al.* 2011; Ng & Tan 2015).

Electrophoresis of ISSR-PCR products generally uses 1.5-2.0% agarose gel by weight/volume (w/v) in order to achieve good DNA band separation. When the agarose gel concentration is higher, reaching 3% w/v or more, the gel will break more easily as it hardens (Ng & Tan 2015). According to Bornet & Branchard (2001), 2.0% w.v agarose gel showed the best performance in resolving the ISSR band compared to other concentrations of agarose gel.

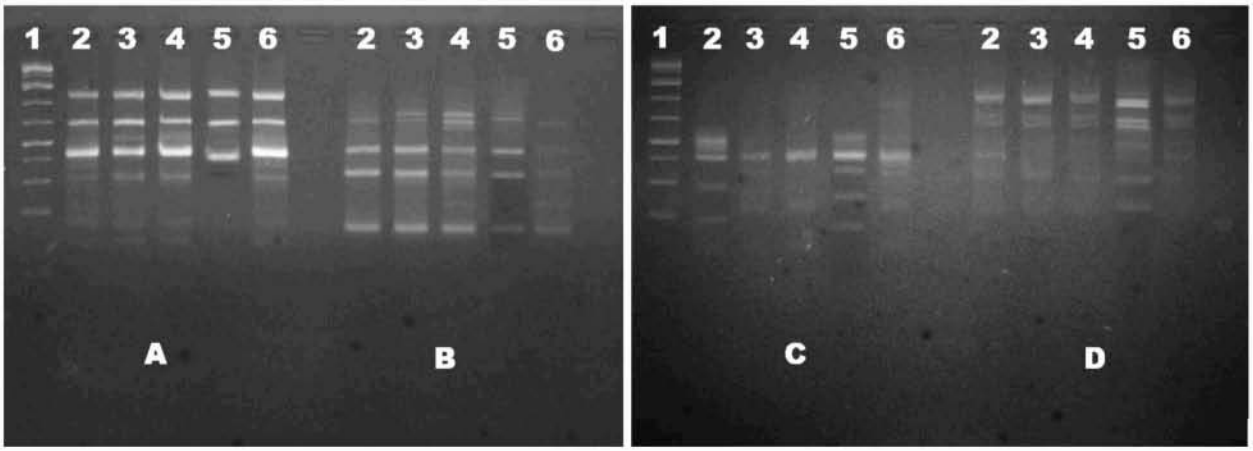

Figure 1 Amplification results
Notes: Amplification with 5 accessions (SENG1 (lane 2); SIPE3 (lane 3); TENG1 (lane 4); KEKE1 (lane 5); and SUBR1 (lane 6)) using ISSR markers (UBC 811 (group A); UBC 818 (group B); UBC 825 (group C); and UBC 827 (group D)) with 100 bp DNA ladder (lane 1).

Plant systematic studies of several plant taxa based on the molecular characteristics using ISSR-PCR revealed a greater number of polymorphic loci than the use of RAPD-PCR, because the abundant formation of ISSR primer target sequences throughout the eukaryotic genome, which evolves rapidly (Ansari *et al.* 2012; Phong *et al.* 2011; Moulin *et al.* 2012). Previous study on genetic variability of arrowroot in Yogyakarta using RAPD markers showed a low polymorphism and required further study (Setyowati 2013). In this recent study, the use of ISSR was proven to show the genetic variability of arrowroot in Yogyakarta. Therefore, ISSR marker proved to be better and more suitable in the analysis of arrowroot genetic variability than RAPD.

ISSR is a recent genetic marker that overcomes many technical limitations in other methods such as RFLP and RAPD analyses. ISSR markers have higher reproducibility than that of RAPD and have been successfully used to estimate the extent of genetic diversity at intra and inter-specific levels in a wide range of crop species (Asha *et al.* 2015). Hence, the ISSR markers were used in our study to assess the genetic diversity of arrowroot.

The amplification results of the 4 primers with 7 arrowroot accessions in India showed high polymorphism values (Asha *et al.* 2015), which were in agreements with the results of this study. However, the number of total DNA bands and the average percentage of polymorphisms obtained were lower than the previous research conducted by Asha *et al.* (2015). The differences were due to genetic diversity among plant population influenced by geographical condition and also appeared as molecular characteristics of each sample. Based on both studies, the polymorphic DNA bands obtained from ISSR analysis ranged from 10 to 60 fragments from various loci.

The use of ISSR UBC 825 primer in this study resulted in the highest polymorphism values and the greatest number of polymorphic DNA bands. This showed the effectiveness and the high reproducibility of these primers in the analysis of genetic diversity of arrowroot plants. The primary use of UBC 825 in the previous study conducted by Asha *et al.* (2015) was also known to produce the greatest number of polymorphic DNA bands and the highest polymorphism. Based on the electrophoresis, UBC 825 indicated the presence of microsatellite DNA sequences with sizes ranging from 800 bp to 180 bp.

Phenetic Analysis Based on Molecular Characteristics

Phenetic analysis based on the molecular characteristics can separate five local cultivars of arrowroot in Yogyakarta into four clusters. Dendrogram branching resulted in four clusters which were determined with the help of phenon lines at 80% similarity index. Three of the four clusters were outliers, each consisted of accessions of SIPE3, TENG1 and KEKE1. One cluster consisted of accessions SENG1 and SUBR1 (Fig. 2).

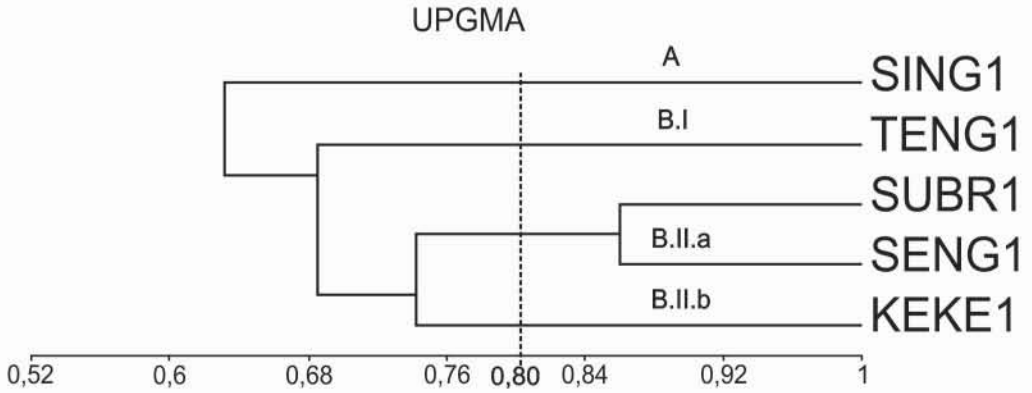

Figure 2 Dendrogram of arrowroot phenetic relationship based on molecular characteristics
Note: Molecular characteristics were the result of DNA fingerprinting analysis with ISSR markers, using the Baroni-Urbani Buser similarity coefficient.

Table 6 Similarity matrix for cluster analysis of arrowroot based on molecular characteristics

	KEKE1	SENG1	SUBR1	TENG1	SING1
KEKE1	1				
SENG1	0.741	1			
SUBR1	0.745	0.86	1		
TENG1	0.698	0.672	0.629	1	
SING1	0.654	0.676	0.726	0.534	1

Dendrogram branching was determined by the similarity matrix between accessions. The similarity values between one accession and the other accessions were different (Table 6). Analysis using the Baroni-Urbani Buser similarity coefficient showed that the accessions of SUBR1 and SENG1 had the highest similarity coefficient, which was 0.86. The lowest similarity coefficient was shown by SING1 and TENG1, which is 0.534.

The dendrogram showed 4 branches (Fig. 2). The first branch separated the SING1 accession, representative of the local cultivar 'Sili', from the other four local cultivar accessions at 0.64 point. It indicated the high dissimilarity between 'Sili' local cultivar with the other five local cultivars based on its molecular genetics. The genotypic characters obviously made distinct phenotypic characters of 'Sili' local cultivars. Based on interviews with arrowroot farmers, it was found that the 'Sili' cultivar was not very attractive for cultivation because it produced rhizomes containing higher fiber and smaller size than the other four local cultivars rhizomes. The second branch of the dendrogram separated the cultivar of 'Teropong' (TENG1) from 'Sugo', 'Sembowo', and 'Kebo' cultivars at 0.68 point. Then, the KEKE1 accession separated from the cluster consisting of SUBR1 and SENG1 accessions at 0.86 point. The highest similarity that showed by 'Sugo' cultivar and 'Sembowo' cultivar was supported by the similarity of rhizome morphologies from both cultivars, which information was provided by the farmers during the interviews. However, around 10% of dissimilarity index obtained based on molecular characteristics still showed great differences on the morphological and or anatomical characters.

Further studies of the use of ISSR markers in DNA fingerprinting techniques, cultivation or revealing genetic variation can provide several advantages. Genotype-specific ISSR markers can be sequenced to be used as a basis for the synthesis of Sequence Characterized Amplified Region (SCAR) primers, which can then be used to determine genotype taxonomy. In addition, markers associated with high agronomic value characters can be sequenced to be used as Sequence Tagged Sites (STS) markers that are useful in cultivation and breeding programs. Meanwhile, although the microsatellites that play a role in ISSR-PCR are probably nonfunctional, they are known to be associated with region coding. Therefore, ISSR can be used to construct gene-rich regions (Vijayan 2005).

CONCLUSION

The study used 5 local cultivars of arrowroot (*M. arundinacea* L.) from four districts in the Yogyakarta Province consisting of five local cultivars namely 'Sembowo', 'Sili', 'Kebo', 'Teropong' and 'Sugo'. Molecular analysis using ISSR markers was successful in showing high polymorphism and explaining genetic variabilities in the five local arrowroot cultivars. The analysis of the phenetic relationship using the MVSP program, the UPGMA algorithm method and the Baroni-Urbani Buser similarity coefficient showed the formation of four clusters on the dendrogram based on molecular characteristics. Clustering based on molecular characteristics was able to separate local cultivars from one another, but showed a close relationship between the local cultivars 'Sembowo' and 'Sugo'.

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REFERENCES

Amani J, Kazemi R, Abbasi AR, Salmanian AH. 2011. A simple and rapid leaf genomic DNA extraction method for polymerase chain reaction analysis. Iran J Biotech 9:69.

Anne C. 2006. Choosing the right molecular genetic markers for studying biodiversity: From molecular evolution to practical aspects. Genetica 127: 101-20.

Ansari SA, Narayanan C, Wali SA, Kumar R, Shukla N, Rahangdale SK. 2012. ISSR markers for analysis of molecular diversity and genetic structure of Indian teak (*Tectona grandis* Lf) populations. Annals of Forest Research 55(1):11-23.

Aprianita A, Vasiljevic T, Bannikova A, Kasapis S. 2014. Physicochemical properties of flours and starches derived from traditional Indonesian tubers and roots. J Food Sci Technol 51:3669-79.

Asha KI, Radhika NK, Vineetha B, Devi AA, Sheela MN, Sreekumar J. 2015. Diversity analysis of arrowroot (*Maranta arundinacea* L.) germplasm using ISSR markers. J Root Crops 14(1):17-24.

Bani PW, Daryono BS, Purnomo. 2017. Penanda molekuler Inter Simple Sequence Repeat untuk menentukan ketahanan tanaman jagung terhadap penyakit bulai. Jurnal Fitopatologi Indonesia 13(4):127-35.

Blair MW, Panaud O, McCouch SR. 1999. Inter-simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and fingerprinting in rice (*Oryza sativa* L.). Theor Appl Genet 98:780-92.

Bornet B, Branchard M. 2001. Nonanchored inter simple sequence repeat (ISSR) markers: Reproducible and specific tools for genome fingerprinting. Plant Mol Biol Rep 19:209-15.

Djaafar TF, Sarjiman, Pustika AB. 2010. Pengembangan budi daya tanaman garut dan teknologi pengolahannya untuk mendukung ketahanan pangan [Development of arrowroot cultivation and its processing technology to support the food security]. Jurnal Litbang Pertanian 29(1):1-10.

Deswina P, Prihastuti L, Saputra A. 2019. Cytomorphological characteristic evaluation of the third generation of arrowroot plant (*Maranta arundinacea* L.) radiated by gamma ray. Jurnal Agro 6(2):181-95.

Faraldo MIF, Silva RM, Ando A, Veasey EA. 2003. Molecular markers in cassava. In: Cereda MP, editor. Agricultura: Culturas de tuberosas amiláceas Latino-Americanas [Agriculture: Cultivation of Latin America Starchy Tubers]. São Paulo: Fundação Cargill. p. 100-17.

Govindaraj M, Vetriventhan M, Srinivasan M. 2015. Importance of genetic diversity assessment in crop plants and its recent advances: an overview of its analytical perspectives. Genetics Research International Volume 2015:1-14.

Guilherme DdO, Branco FP, Madeira NR, Brito VH, de Oliveira CE, Jadoski CJ, Cereda MP. 2017. Chapter 5 starch valorization from corm, tuber, rhizome, and root crops: the arrowroot (*Maranta arundinacea* L.) case. In: Clerici MTPS, Schmiele M, editors. Starches for Food Application: Chemical, Technological, and Health Properties. Cambridge (US): Academic Press. p. 167-222.

Gupta SK, Souframanien J, Gopalakrishna T. 2008. Construction of a genetic linkage map of black gram, *Vigna mungo* (L.) Hepper, based on molecular markers and comparative studies. Genome 51: 628-37.

Hammel BE, Grayum MH, Herrera C, Zamora N. 2014. Manual de Plantas de Costa Rica [Costa Rica Plant Manual]. Missouri (US): Missouri Botanical Garden Press. p 78-80.

Heredia-Zárate NA, Vieira MC. 2005. Produção da araruta 'comum' proveniente de três tipos de propágulos [Production of the 'comum' arrowroot from three types of propagules]. Ciência e Agrotecnologia 29(5):995-1000.

Horne EC, Kumpatla SP, Patterson MG, Thompson SA. 2004. Improved high-throughput sunflower and cotton genomic DNA extraction and PCR fidelity. Plant Mol Biol Rep 22:83.

Kitahara K, Hamasuna K, Nozuma K, Otani M, Hamada T, Shimada T, Fujita K, Suganuma T. 2007. Physicochemical properties of amylose-free and high-amylose starches from transgenic sweet potatoes modified by RNA interference. Carbohyd Polym 69:233-40.

Koetsier G, Cantor E. 2019. A practical guide to analyzing nucleic acid concentration and purity with microvolume spectrophotometers [Technical Notes] New England (US): BioLabs Inc.

Lestari LA, Huriyati E, Marsono Y. 2017. The development of low glycemic index cookie bars from foxtail millet (*Setaria italica*), arrowroot (*Maranta arundinacea*) flour, and kidney beans (*Phaseolus vulgaris*). J Food Sci Technol 54:1406-13.

Li JT, Yang J, Chen DC, Zhang XL, Tang ZS. 2007. An optimized mini-preparation method to obtain high-quality genomic DNA from mature leaves of sunflower. Genet Mol Res 6:1064.

Lim TK. 2016. Edible Medicinal and Non-Medicinal Plants: Volume 11, Modified Stems, Roots, Bulbs. Switzerland (CH): Springer International Publishing. p. 47-54.

Madineni MN, Faiza S, Surekha RS, Ravi R, Guha M. 2012. Morphological, structural, and functional properties of *Maranta* (*Maranta arundinacea* L.) starch. Food Sci Biotech 21(3):747-52.

Masitoh S. 2014. Densitas spesies garut (*Maranta arundinacea*) di Daerah Istimewa Yogyakarta [Density of arrowroot (*Maranta arundinacea* L.) in Special Region of Yogyakarta] [Undergraduate Thesis]. Yogyakarta (ID): Universitas Islam Negeri Sunan Kalijaga.

Mason W. 2009. Starch use in foods. In: Whistler RL, BeMiller JN, Paschall EF, editors. Starch: Chemistry and Technology, 3rd ed. New York: Academic Press. p. 1-879.

Mommaerts K, Sanchez I, Betsou F, Mathieson W. 2015. Replacing b-mercaptoethanol in RNA extractions. Anal Biochem 479:51-3.

Moulin MM, Rodrigues R, Gonçalves LSA, Sudré CP, Pereira MG. 2012. A comparison of RAPD and ISSR markers reveals genetic diversity among sweet potato landraces (*Ipomoea batatas* (L.) Lam.). Acta Sci Agron 34(2):139-47.

Ng WL, Tan SG. 2015. Inter-Simple Sequence Repeat (ISSR) Markers: Are We Doing It Right? . ASM Sci J 9(1):30-9.

Perez-de-Castro AM, Vilanova S, Cañizares J, Pascual L, Blanca, JM, Diez MJ, Prohens J, Picó B. 2012. Application of genomic tools in plant breeding. Current Genomics 13:179-95.

Phong DT, Hien VTT, Thanh TTV, Tang DV. 2011. Comparison of RAPD and ISSR markers for assessment of genetic diversity among endangered rare *Dalbergia oliveri* (Fabaceae) genotypes in Vietnam. Genet Mol Res 10(4):2382-93.

Pinto IB. 2015. Caracterização dos genes matK e rbcL e da variabilidade genética entre os acessos de Araruta (*Maranta arundinacea* L.) [Characterization of matK and rbcL genes and genetic variability among Araruta (*Maranta arundinacea* L.) accessions]. Compo Grande (BR): Universidade Católica Don Bosco. p. 60.

Reddy MP, Sarla N, Siddiq EA. 2002. Inter simple sequence repeat (ISSR) polymophism and its application in plant breeding. Euphytica 128:9-17.

Rohlf FJ. 2000. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, version 2.1. Exeter Software. New York (US): Setauket.

Sambrook J, Russell DW. 2001. Molecular Cloning. A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press.

Setyowati N. 2013. Tanaman garut (*Maranta arundinacea* L.) lokal DI Yogyakarta [Local Plant of Arrowroot (*Maranta arundinacea* L.) in DI Yogyakarta] [Thesis]. Yogyakarta (ID): Universitas Islam Negeri Sunan Kalijaga.

Shafiei-Astani B, Ong AHK, Valdiani A, Tan SG, Yong CSY, Ahmady F, Alitheen NB, Ng WL, Kaur T. 2015. Molecular genetic variation and structure of Southeast Asian crocodile (*Tomistoma schlegelii*): Comparative potentials of SSRs versus ISSRs. Gene 571:107-16.

Shen J, Ding X, Liu D, Deng G, He J, Li X, Tang F, Chu B. 2006. Intersimple sequence repeats (ISSR) molecular fingerprinting markers for authenticating populations of *Dendrobium officinale* Kimura et Migo. Biological and Pharmaceutical Bulletin 29:420-22.

Silva JR, Assumpção R., Vegro CLR. 2000. A inserção da fécula de mandioca no mercado de amido [Insertion of cassava starch in the starch market]. Informações econômicas 30(7):31-41.

Silveira JRS, Santos CMFT, Silva JB, Batista AJ, Costa JA. 2013. Resgate da cultura da araruta junto aos agricultores familiares no Território do Recôncavo da Bahia [Arrowroot crop rescue from family farmers in the Reconcavo da Bahia Territory]. Cadernos de Agroecologia 8(1):1-5.

Suman PSK, Ajit KS, Darokar MP, Sushil K. 1999. Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Mol Biol Rep 17:1.

Tautz D, Renz M. 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Res 12(10):4127-38.

van Steenis CGGJ. 1975. Flora: untuk sekolah di Indonesia [Flora: for school in Indonesia]. Jakarta (ID): Pradnya Paramita. p. 167.

Vijayan K. 2005. Inter Simple Sequence Repeat (ISSR) polymorphism and its application in mulberry genome analysis. Int J Indust Entomol 10:79-86.

Wang HZ, Wu ZX, Lu JJ, Shi NN, Zhao Y, Zhang ZT, Liu JJ. 2009. Molecular diversity and relationships among *Cymbidium goeringii* cultivars based on inter-simple sequence repeat (ISSR) markers. Genetica 136(3):391-9.

Wolfe AD, Xiang QY, Kephart SR. 1998. Diploid hybrid speciation in *Penstemon* (Scrophulariaceae). Proc Natl Acad Sci 95:5112-5.

Wolff K, Zietkiewicz E, Hofstra H. 1995. Identification of *Chrysantemum* vultivars and stability of DNA fingerprint patterns. Theor Appl Genet 91:439-47.

Woodson RE, Schery RW. Flora de Panama [Flora of Panama]. Ann Missouri Bot 67(4):ii-xxxii.

Wu D, Kennedy H. 2000. Marantaceae. In: Wu ZY, Raven PH, editors. Flora of China, Vol 24, Flagellariaceae through Marantaceae. Beijing (CN)/St. Louis (US): Science Press/Missouri Botanical Garden Press. p. 27-30.

Zietjiewicz E, Rafalski A, Labuda D. 1994. Genome fingerprinting by simple sequence repeat (SSR): Anchored polymerase chain reaction amplification. Genomics 20(1):176-83.

INCORPORATION OF SODIUM HYALURONATE AND NYAMPLUNG (*Calophylum inophyllum*) CAKE EXTRACT TO IMPROVE BIOPLASTIC CHARACTERISTIC

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ABSTRACT

The cross-linking agent plays an important role in bioplastic mechanical properties. This study aimed to determine the effect of Sodium Hyaluronate (SoHA) as a synthetic cross-linking agent and the addition of Nyamplung Cake Extract (NCE) as an antimicrobial agent in the manufacture of bioplastic with hydroxypropyl starch (HPS) as based ingredient using the thermo-compression method. The novelty of the study was thiocyanate (SCN) formation in zone 3 (2161.66-2162.02/cm) and cyanate (C-N=O) in zone 6 (1,411.57-1,412.61/cm) of (1, 2 and 3%) SoHA bioplastic and cyanate formation in zone 6 and 7 (1,411.37-1,558.59/cm) of (1, 2 and 3%) SoHA-20% NCE combined bioplastic originating from acetanilide group in SoHA and amide group in NCE. The formation of SCN and C-N=O in 2 and 3% SoHA bioplastic improved its sensitivity against gram-positive bacteria (*Staphylococcus aureus*) indicated by 0.6 mm and 0.45 mm inhibition zone, respectively. C-N=O formed in (1, 2 and 3%) SoHA-20% NCE combined with bioplastic had 3.25 mm average inhibition zone against gram-positive bacteria (*S. aureus*), 2.75 mm against gram-negative bacteria (*Escherichia coli*), and 0.71 mm against fungi (*Aspergillus niger*). The analysis of mechanical properties showed that an addition of 3% SoHA was able to increase tensile strength and modulus of elasticity while reducing elongation, water solubility and water vapor permeability. Addition of (1, 2 and 3%) SoHA-20% NCE resulted in a reverse effect.

Keywords: acetanilide, amide, cross-linking agent**,** cyanate, thiocyanate

INTRODUCTION

Natural polymers, such as starch, have various potential applications to replace commercial market-dominant petroleum-based plastics due to their abundant availability, degradability, renewability and low price (Samsudin & Hani 2019). The most critical property of plastic is its ability to be adjusted for specific purposes. The increasing utilization of starch for commercial use, such as to produce biodegradable thermoplastic, is expected to reduce problems caused by the petroleum-based polymer in solid waste disposal (Manoi & Rizvi 2010). As a renewable polymer source with film-forming ability, starch fulfills several requirements as an alternative for plastic, such as having abundant availability, high extraction yield, cheap, biodegradable and biocompatible. Starch-based bioplastic is also odorless, tasteless, colorless, non-toxic, and semi-permeable to carbondioxide, temperature, oxygen, as well as fat and aroma components (Shah *et al.* 2016).

Starch-based bioplastic as an environmentally friendly package has various disadvantages related to the hydrophilic and hygroscopic properties of starch. In this study, hydroxypropyl starch (HPS) as a modified starch was used as the main material, because HPS is more feasible to be processed into bioplastic due to its lower solubility temperature, with more transparent and flexible plastic properties (Woggum *et al.* 2015). Therefore, the mechanical properties of bioplastic can be improved and

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adjusted by adding a plasticizer or cross-linking agent. A plasticizer is a group of small molecules that are able to blend and interact among polymer chains by intermolecular force, thus reduces glass transition temperature of a material to improve malleability, flexibility, extension and to soften the texture (Wypych 2017). On the other hand, cross-linking mechanism works by formating bridges of immediate intermolecular bond by chemicals known as cross-linking agent. Cross-linking means polymer molecules are interconnected by bond (Canisag 2015). Cross-linking agent is substances that are able to form permanent chemical bond among polymer chains, to increase material rigidity, firmness, strength, less flexible and increase glass transition temperature (Tg). Cross-linking agent might affect permeability property, thermal stability, glass transition temperature and the rate of wear (Frost *et al.* 2013) due to formation of covalent or ionic bond connecting a polymer chain to the others which enhance polymer strength.

Cross-linking process is critical to modify starch properties by enhancing the starch's intra- and inter-molecular bonds in random patterns. The bonds tend to inhibit starch interaction to water while bringing structural integrity on starch-based biodegradable materials during hydraulic pressure and high humidity. Cross-linking process can be done by starch treatment, both in semi-solid or pulp form, using reagent with ability to form ether or esther bond among hydroxyl (-OH) in starch molecule (Manoi & Rizvi 2010). Cross-linking agents are able to form cross bond that enhances matrix density and tensile strength, and even permanent chemical bond among polymer chains, thus generate more rigid, strong, less flexible material and increase glass transition temperature (Tg).

Polyfunctional chemicals such as phosphor oxychloride ($POCl_3$), sodium trimetaphosphate (STMP), sodium tripolyphosphate (STPP), epiclorohydrine (EPI), a mixture of adipic, acetate anhydride, and mixture of succinic anhydride and vinyl acetate are some of the common cross-linking agents for starch. In this study, we used sodium hyaluronate (SoHA) as a cross-linking agent. SoHA is a polymer of disaccharides, consisting of D-glucuronic acid and D-N-acetyglucosamine, linked by β-1, 4 and β-1, 3 bonded by glycosidicbonds, into polymer compounds that do not have UV absorbing chromophores (Ruckmani *et al.* 2013).

Hyaluronate supports the structure of connective tissue by acting as a magnet to maintain fluidity and form viscous liquids with lubrication properties. SoHA has low toxicity and no report of negative effect on human (Spec-Chem Ind. 2014). SoHA is also suitable as cross-linking agent and encapsulates various active ingredients (Contipro 2018).

This study used nyamplung cake extract (NCE) as antimicrobial agent because of the ability of NCE against gram-positive bacteria (*Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*). Bioplastic is more sensitive against gram-positive bacteria compared to gram-negative bacteria. Bioplastic has the largest inhibition zone of 30 mm against *Staphylococcus aureus* and that of 23 mm against *Escherichia coli.* The abiltiy of bioplastic as antimicrobial agent might be related to the content of the bioplastic extract which serves as natural cross-linking and antimicrobial agents (Umiyati *et al.* 2019).

The purpose of this study was to determine the effect of SoHA as synthetic cross-linking agent and nyamplung cake extract (NCE) as antimicrobial agent in the manufacture of bioplastic with HPS as basic ingredient using thermo-compression method. This study demonstrated that the features of bioplastic produced showed better mechanical properties, having antimicrobial ability compared to the conventional bioplastic.

MATERIALS AND METHODS

Materials

Nyamplung cake (*Calophyllum inophyllum* L.) was obtained from biodiesel industry in Purworejo, Central Java, Indonesia. Nyamplung cakes were sorted and dried until reaching moisture content below 10-15%. The dried cakes were finely ground and sifted using a 120-mesh sieve to obtain nyamplung cake powder. Subsequently, 20 g of the nyampung cake powder was extracted by using 120 mL of 96% ethanol and placed on a hot plate stirrer at 80 °C for 1 h. The mixture was filtered using Whatman filter paper No. 1. Based on the optimal method

by Chana-Thaworn *et al.* (2011), the extracted liquid was then evaporated and ovendried at 50 °C for 24 h. Other materials used in this study were hydroxypropil starch (HPS), sodium hyaluronate (SoHA), glycerol 20%, etanol 96% and distilled water.

Instruments

Thermo-compression machine, FTIR (Thermo Scientific Nicolet iS10), Scanning Electron Microscopy (JEOL JSM 6510) and texture analyzer (Brookfield USA) were used in this study.

Bioplastic Preparation

Various concentrations of SoHA (1%, 2% and 3% b/b) were mixed with 10 g of HPS, 2 g of nyamplung cake extract, 1.5 g of glycerol and 1 g of distilled water, then mixed at 21,000 rpm for 45 min until homogen. The mixture was then made into bioplastic using thermo-compression mold. The mixture was then placed in the middle of a 0.5 mm-thick aluminum frame sized 10 × 10 cm that was put between the two previously heated aluminum plates, molded at 140 °C and 250 kg/m^2 pressure for 6 min in heated hydrolic pressure machine (Rasheed *et al.* 2015).

Fourier Transform Infrared Spectroscopy (FTIRs) analysis is a method to identify structural changes in starch chains due to interaction among extract, glycerol, NaHA, and HPS molecules (Bilal *et al.* 2015). FTIR spectra of bioplastic was measured by Thermo Scientific Nicolet iS10, equipped with Smart ATR Diamond at an area of 650-4,000/cm using 32 scanning and resolution of 8/cm.

Scanning Electron Microscopy (SEM) was used to analyze microplastic structure of the bioplastic. Sample of bioplastic was stored in a desiccator with P_2O_5 absorbent for two weeks to ensure no moisture left in the bioplastic sample. For cross-section observation, the bioplastic sample was frozen in N_2 liquid for cryofracture preservation (Espinel *et al.* 2014). All samples were placed in bronze stub and covered with gold sheets before imaging. Micrograph of the surface of bioplastic and fracture was obtained using SEM (JSM-6510LA) at 10-300,000x magnification and 1-10 nm resolution.

Characterization of Bioplastic Physical Properties

Mechanical properties such as tensile strength (σ), elongation (ε), modulus of elasticity (Y), water solubility (WS) and water vapor permeability (WVP) were measured based on the methods in ASTM D638. Prior to mechanical properties analysis, the bioplastic sample was conditioned at RH of 50 ± 5 and 23 °C for 48 h in humid chamber. Tensile strength at room temperature was measured using Brookfiled USA analyzer texture. Bioplastic specimen was cut according to the method by ASTM D1708 at a constant pull rate of 5 mm/minute. Solubility measurement of bioplastic sample in water was conducted by drying the bioplastic sample at 105 °C and then weighed as m3. Subsequenty, the bioplastic sample was soaked in 50 mL distilled water at 24 °C for 6 h, then ovendried at 105 °C for 24 h and weighed as m4 (Hassannia-kolaee *et al.* 2016; Kumari *et al.* 2017), before calculation using formula below (1):

$$WS = \frac{m3 - m4}{m4} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where: m3 = weight of bioplastic sample before drying
m4 = weight of bioplastic sample after drying

Water vapor permeability (WVP) was analyzed using standard method in ASTM (1996) E96. The bioplastic sample was placed as a cover on circle permeation cells sized 4 cm in diameter containing silica gel (0% RH) and placed in a desiccator which was previously filled with saturated sodium chloride liquid (75% RH), then stored at 30 °C. Water vapor permeability rate was measured by permeation cell weighing at a frequency of every 30 min to obtain several points. The WVP was counted using a formula (2) developed by Wirawan *et al.* (2012):

$$WVP = \frac{WPTR}{Ps\,(RH_1 - RH_2)} \times \delta m \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where:
WVP = water vapor permeability (g.mm/KPa.s.m^2)
WVTR = mass increase (g)
Ps = pressure of saturated water vapor (Pa)

RH_1 = relative humidity in desiccator
RH_2 = relative humidity in permeation cell
δm = average thickness of bioplastic layer (m)

Characterization of Bioplastic Antimicrobial Properties

Disk agar method was used for analyzing the antimicrobial activity of bioplastic sample using distilled water as solvent. As much as 0.1 mL of 10% bacteria and fungi suspension was inoculated on Mueller Hinton Agar and on Potato Dextrose Agar media, respectively. Bioplastic sample of 1 cm diameter was placed on a sterile disk sized 6 mm diameter and let to dry. Subsequently, the bioplastic sample was placed on the surface of culture media in a Petri dish that was previously inoculated with tested microorganisms, then incubated at 37 °C for 1-2 days for bacteria and at 30 °C for 2-3 days for fungi, based on optimum condition reported by Chanwitheesuk *et al.* (2007).

RESULTS AND DISCUSSION

Bioplastic Characteristics

Mechanical Properties

Comparison of mechanical properties in the form of tensile strength (σ), elongation at break (ε) and modulus of elasticity (Y) of bioplastic prepared with (1, 2 and 3%) SoHA, combination of (1, 2 and 3%) SoHA-20% NCE compared to that prepared by using 20% NCE and control is presented in Figure 1. Those mechanical parameters depend on microstructure characteristics (Aguirre *et al.* 2013).

Bioplastic thickness ranged from 0.24 to 0.32 mm (data not shown). Results of this study showed that the control bioplastic had significant increase of tensile strength (σ) compared to 3% SoHA bioplastic and 20% NCE bioplastic, by 23.7% and 50.8%, respectively, while that of (1, 2 and 3%) SoHA-20% NCE bioplastic had significant reduction by an average of 62.4% (Fig 1). In terms of elongation at break (ε), the (2 and 3%) SoHA bioplastic showed significant reductions compared to the control bioplastic by an average of 32.4%, while the (1, 2 and 3%) SoHA-20% NCE bioplastic had a significant increase of 224.2%. For modulus of elasticity (Y), there was significant difference between the control and (1, 2 and 3%) SoHA, (1, 2 and 3%) SoHA-20% NCE combination, and 20% NCE. This indicated that SoHA addition to 3% could function as effective cross-linking agent to improve bioplastic mechanical properties. SoHA works to facilitate esterification of starch OH groups in overcoming the poor polymer compatibility by incorporating compatible substances (Ortega-toro *et al.* 2014).

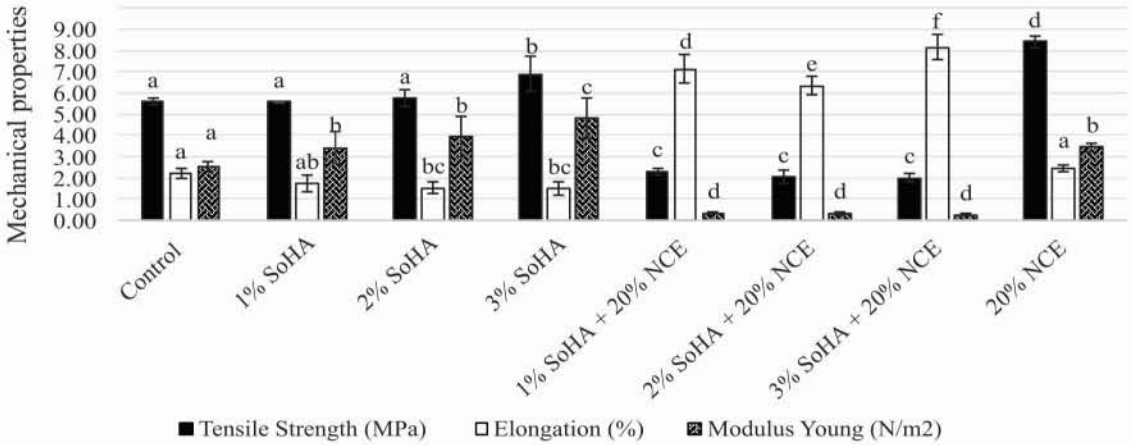


Figure 1 Effect of SoHA addition and combination of SoHA-NCE on the mechanical properties of HPS bio-plastic
Notes: Bar indicates mean ± standar deviation. Different letters in the same column indicate significant differences ($P < 0.05$).

SoHA is a widely distributed natural mucopolysaccharide polymer, which in mammals is known as connective tissue. Cross-linking process is done by SoHA to increase the *in vivo* retention time, so that the hydroxyl groups within the polymer chains bind to one another. The cross-linking reaction in SoHA is carried out in a homogeneous aqueous alkaline solution which can enhance anti-biodegradation ability by increasing the degree of cross-linking and extending the *in vivo* retention time (Xuejun *et al.* 2015).

Utilization of the modified HPS starch as basic material may also serve to improve bioplastic mechanical properties due to its high amylose content of modified starch. Therefore, HPS is feasible as bioplastic raw material, has mechanical strength and functions as good oxygen inhibitors (Woggum *et al.* 2015). Concentration of cross-linking agent, pH, treatment temperature and storage period might determine cross-linking bond. Reaction condition can be varied according to the type of cross-linking agent (Canisag 2015).

Utilization of SoHA-NCE combination reduced σ and Y. Higher concentration of SoHA on NCE resulted in a lower σ. These effects were probably due to the loss of amide group in NCE bioplastic (20% extract) by the combination. On the other hand, SoHA-NCE combination increased ε, which probably due to H_2O formation from reactions involving SoHA and hydroxyl groups from HPS starch, glycerol-SoHA and amide groups of NCE-SoHA. C-N=O is an intermediate product of the first stage of thiocyanate hydrolysis, which was then hydrolyzed into ammonia and bicarbonate (Doble & Kumar 2005).

Stronger interaction of HPS-SoHA-glycerol reduces Y and maximum tension as well as tension-when-separate resulted in a more flexible bioplastic, and therefore, showing the function of SoHA as cross-linking agent (Contipro 2018). Modified starch was reportedly a more stable raw material for making bioplastic compared to native starch (Gutiérrez *et al.* 2015). Bioplastic preparation might also play a role as thermo-compression utilizes high temperature and hydroulic pressure. There was also the possibility of maillard reaction which can form more compact networks (Leceta *et al.* 2013).

HPS as raw material might affect bioplastic mechanical properties due to the effect of hydroxypropylation that interfere the inter- and intra-molecular hydrogen bond of the starch chain. The effect can enervate starch granule while increasing starch chain movement in an amorph area and enhancing HPS swelling power compared to native starch with increasing molar substitusion (Woggum *et al.* 2015). SoHA addition brought significant increase on σ at ≥ 3%, but NCE addition in SoHA bioplastic did otherwise.

Water Solubility (WS)

SoHA and SoHA-NCE combination affected bioplastic water solubility (WS) in terms of bioplastic integrity in watery media. High solubility of bioplastic indicates low water resistency (Gutiérrez *et al.* 2015). WS is defined as the ratio of dry matter in water, such as bioplastic soaked in distilled water (Hassannia-kolaee *et al.* 2016). Results of this study showed that WS of bioplastic made by using SoHA, combination of SoHA-NCE and NCE were significantly lower than that of control (Fig. 2).

SoHA bioplastic when compared to control had an average of lower WS values by 6.6%, while bioplastic made by using SoHA-NCE combination had an average of lower values by 15.9%, then bioplastic made by using NCE had an average of lower values by 20.95%. Insignificant WS decrease was observed in 2% SoHA compared to 3% SoHA bioplastics and vice versa, as well as in 1% SoHA-20% NCE combination compared to NCE bioplastics (Fig. 2). This showed that NCE addition affected the molecular structure of SoHA bio-plastic.

During bioplastic preparation, phosphor of STMP (as a cross-linking agent in making modified starch) reacted with hydroxyl (-OH) group of starch to form phosphate distarch (cross-bond) and other phosphate derivates as indicated by the FT-IR results. Cross-linking process enhances granule structure starch and inhibit both water absorption and starch solubility, thus limit the mobility of starch chain in amorph area (Manoi & Rizvi 2010). Bioplastic prepared by HPS-SoHA had lower solubility compared to control, with increasing concentration of cross-linking agent. This

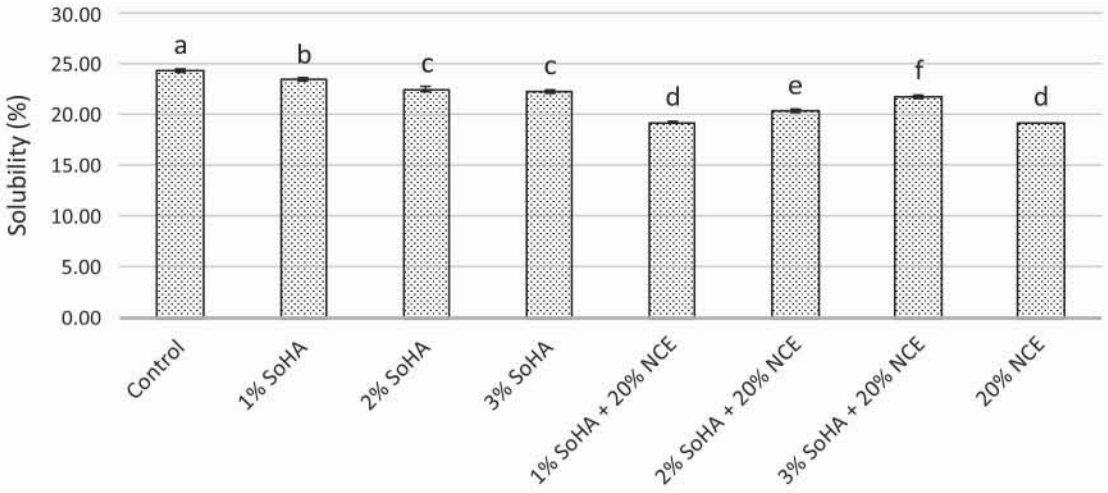


Figure 2 Effect of SoHA addition and combination of SoHA-NCE on the water solubility of HPS bio-plastic
Notes: Bar indicates mean ± standar deviation. Different letters in the same column indicate significant differences ($P < 0.05$).

finding was comparable to previous study conducted by (Manoi & Rizvi 2010) which indicated that the formation of phosphate distarch act as cross-linking agent might limit swelling and hydration of starch granules. Hyaluronate is known as a hydrophilic polymer derivative polysaccharide that has ability as an enhancer of percutaneous penetration by changing composition of cells of tightly arranged materials into more tenuous so that permeability is increased. Permeability has an inverse relationship with WS so that the addition of SoHA can decrease WS of bioplastics (Djajadisastra *et al.* 2014). WS is an important property of bioplastic for food prevention application, particularly in high water activity, or when bioplastic must be in contact with water, such as during food processing. Generally, high solubility indicates lower water endurance, though high solubility might be advantageous for some applications (Chana-Thaworn *et al.* 2011). According to (Spec-Chem Ind. 2014), SoHA can function as a lubricant and film maker, where SoHA is a high molecular weight polymer which is a strong lubricant and film maker.

SoHA as cross-linking agent can be observed from its ability to reduce the WS of bioplastic. It was contrary to the results obtained from SoHA-NCE that increased WS due to water formation from reaction between HPS, SoHA and NCE, showing the hydrophilic property of bioplastic.

Water Vapor Permeability (WVP)

WVP is a bioplastic ability to withold penetrating water vapor and provides information on water vapor transmission through bioplastic as a critical property of food packaging (Leceta *et al.* 2013). Bioplastic permeability is determined by the difference of water vapor concentration between one-side to the other side of bioplastic, with higher difference indicating faster mass transfer and also affected by bioplastic thickness. The results of WVP analysis showed that the control bioplastic had significant difference compared to bioplastic prepared by using 3% SoHA, SoHA-NCE combination and NCE. Increasing concentration of 3% SoHA and 20% NCE bioplastic was able to decrease WVP by an average of 3.66% and 3.89%, respectively, while (1, 2 and 3%) SoHA-20% NCE combination on the contrary increased WVP by an average of 8.25% (Fig. 3).

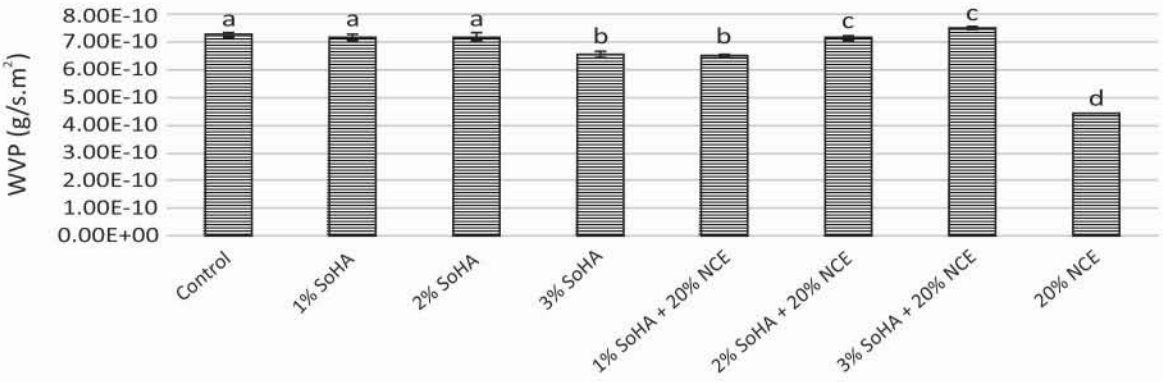


Figure 3 Effect of SoHA addition and combination SoHA-NCE on the WVP of HPS bioplastic
Notes: Bar indicates mean ± standar deviation. Different letters in the same column indicate significant differences ($P < 0.05$).

The results of WVP analysis showed that the control bioplastic had significant difference compared to bioplastic prepared by using 3% SoHA, SoHA-NCE combination and NCE. Increasing concentration of 3% SoHA and 20% NCE bioplastic was able to decrease WVP by an average of 3.66% and 3.89%, respectively, while (1, 2 and 3%) SoHA-20% NCE combination on the contrary increased WVP by an average of 8.25%. Previous study conducted by Gutiérrez *et al.* (2015) reported a tendency of increasing WVP and WS in bioplastic containing native starch, which indicated higher rate of hydrophilic property. Another possibility for increasing WVP in (1, 2 and 3%) SoHA-20% NCE bioplastic was by implementing higher hydrophilic property in the (1, 2 and 3%) SoHA-20% NCE bioplastic compared to (1, 2 and 3%) SoHA and 20% NCE bioplastic.

Water holding capacity of SoHA is very high compared to other moisturizers. The constanta of SoHA moisture evaporation rate is lower than that of other moisturizers. This shows that SoHA has strong water retention properties, with moisture evaporation rate constanta of $8.0 \pm 0.1 \times 100^{-2}$/min (Spec-Chem Ind. 2014). The main function of bioplastic or edible film is to inhibit vapor transfer from the surrounding environment to the food covered by the bioplastic, or between two different components of food products. Therefore, WVP value needs to be as low as possible (Chana-Thaworn *et al.* 2011). WVP is also determined by bioplastic thickness as well as by the glycerol and starch concentrations. Thickness of hydrophilic film might determine WVP, with higher thickness resulted in higher resistance against mass transfer and increasing partial water pressure in equilibrium of film inner surface, thus enhance water vapor permeability of film hydrophilic property with higher thickness (Gutiérrez *et al.* 2015). The enhancing WVP is caused by the changes of partial water vapor pressure of the exposed inner surface of the film.

Separate utilization of SoHA and 20% NCE was able to reduce WVP, indicating their function as cross-linking agent due to their ability to inhibit water vapor penetrating the bioplastic. On the other hand, different results occurred in bioplastic made from a combination of (1, 2 and 3%) SoHA-20% NCE due to deteriorated cross-linked bond from water formation which subsequently caused bioplastic became hydrophilic.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The recent development of material technique has resulted in demand increase for fast, reliable and non-destructive analytical methods to control preparation process and physicochemical characterization. FTIR provides general information on chemistry of the surface and overall characters as well as their functions in polymer (Ricci *et al.* 2015). FTIR spectra of control, (1, 2 and 3%) SoHA, (1, 2 and 3%) SoHA-20% NCE and 20% NCE bioplastic was presented to measure the results of reaction involved in bioplastic preparation made from HPS with SoHA as cross-linking agent, as well as combination of SoHA-NCE (Fig. 4).

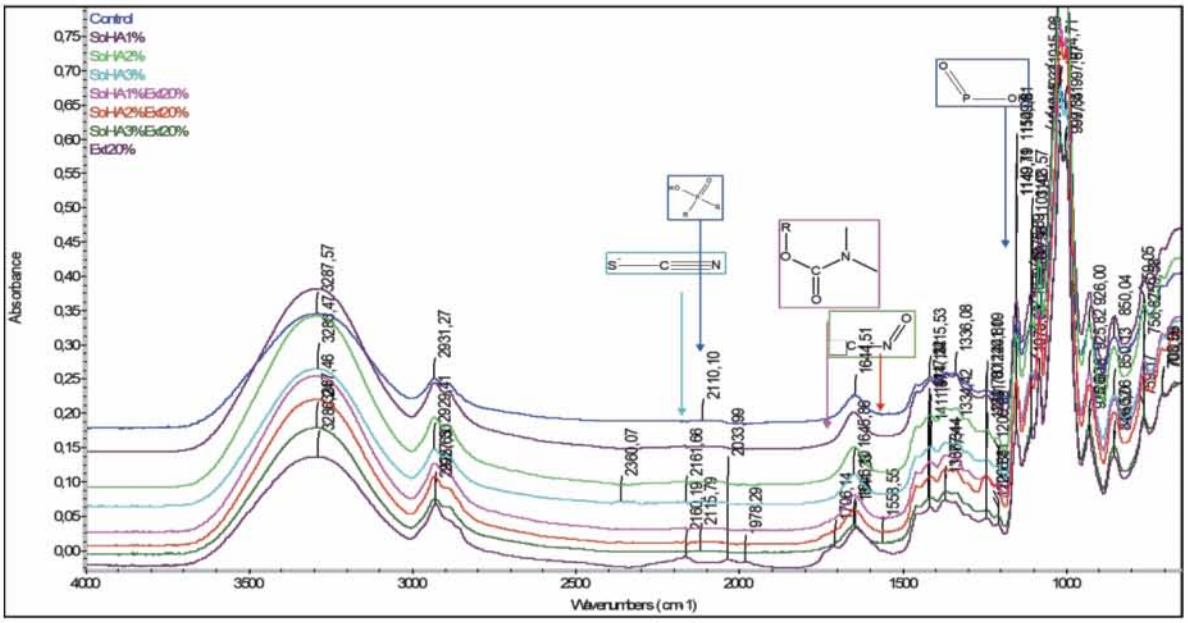


Figure 4 FTIR Spectra of control, (1, 2 and 3%) SoHA, (1, 2 and 3%) SoHA-20% NCE and 20% NCE bioplastics

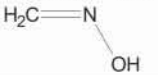
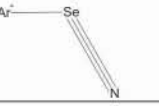
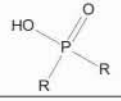
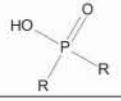
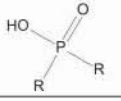
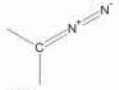
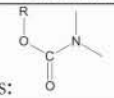
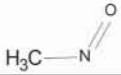
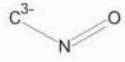
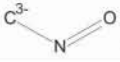
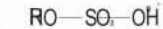
Differences in several peaks of bioplastic made by using 20% NCE compared to (1, 2, and 3%) SoHA and combination of (1, 2 and 3%) SoHA-20% NCE (Fig 4; Table 1). Zone 1 representing bioplastic made by using SoHA at 3,285.91-3,288.09/cm; SoHA-NCE at 3,287.46-3,297.68/cm; and NCE at 3,282.13/cm showed OH bond vibration, related to bound, free, inter- and intra-molecular hydroxyl group (Bilal *et al.* 2015). Zone 2 representing bioplastic made by using SoHA at 2,926.41-2,927.94/cm; SoHA-NCE at 2,927.28-2,927.61/cm; and control at 2,924.49/cm indicated stretching vibration of ketones (CH_3-CO). Zone 3 representing bioplastic made by using SoHA at 2,161.66-2,162.02/cm and 1% SoHA-20% NCE showed stretching vibration of SCN (thyocianates). SCN can be derived from cyanide-rich plants, such as cassava, sweet potato, corn, sugar cane, sorghum and linseed (Chandler & Day 2012).

SCN presence in (1, 2, and 3%) SoHA bioplastic was probably derived from corn starch as HPS raw material. SCN functions as host defense and as cyanide detoxification product. SCN is a preferable substrate for lipid peroxidation (LPO), for catalytic reduction caused by hydrogen peroxyde (H_2O_2) into hypocyanate acid (HOSCN) (Chandler & Day 2012). Eighty percent (80%) SCN in the body comes from cyanide absorbed from food which transformed into SCN in metabolism (Simeonova & Fishbein 2004). In addition, the presence of SCN vibrations may originate from reaction between acetanilide group in SoHA and sulfate compounds from HPS.

Zone 4 representing (2% and 3%) SoHA and (1, 2, and 3%) SoHA-20% NCE bioplastic showed OH-P=O bond area, which arguably indicated cross-linking activity through transformation of starch granule by bifunctional or multifunctional reagents which were able to form ether or ester bond with hydroxyl group of starch (Gui-jie *et al.* 2006). In zone 8 (1,411.37-1,558.59/cm) of (1, 2 and 3%) SoHA-20% NCE bioplastic and zone 9 (1,411.57- 1,412.61/cm) of (1, 2 and 3%) SoHA bioplastic, cyanate (C-N=O) was formed. Whereas, in the 20% NCE bioplastic at 1,704.17/cm, amide group was present. This indicated that NCE combined with (2 and 3%) SoHA removed SCN vibration. Besides hydroxyl group and glycerol, amide group from NCE and acetanilide from SoHA also played important roles in determining bioplastic mechanical properties as well as cyanate (C-N=O) formation observed in peak (1,558.46-1,558.59) (Fig. 4; Table 1).

There was a higher presence of C-N=O in (1, 2 and 3%) SoHA-20% NCE compared to that in (1, 2 and 3%) SoHA bioplastic which probably caused by NCE addition, in dose-dependent manner with the decreasing cyanide concentration due to oxidation. Cyanide can be photocatalytic oxidized into C-N=O,

Table 1 Assigment FTIR Spectra of (1, 2 and 3%) SoHA, (1, 2 and 3%) SoHA-20% NCE and 20% NCE bioplastics

Peaks	SoHA1%	SoHA2%	SoHA3%	Assignment	NCE-1% SoHA	NCE-2% SoHA	NCE-3% SoHA	Assignment	Ext20%	Assignment
1	3285.91	3288.09	3286.47	v: OH stretching vibration Cellulose	3297.68	3296.74	3287.46	v: OH stretching vibration Cellulose	3299.94	v: OH str. Vib 
2	2927.95	2929.50	2926.41	w: CH_3	2927.28	2927.61	2927.30	w: CH_3	2924.49	w: CH_3
3	2162.02	2161.91	2161.66	s-m: CN stretching vibration  thiocyanates	2162.29			s-m: CN stretching vibration  thiocyanates	2159.83	s: aromatic selenocyanates 
4		2114.74	2114.90	w-m: broad 	2115.05	2115.65	2115.79	w-m: broad 	2159.83	w-m: broad 
5									2034.31	v: broad, asym. CNN stretching vibration  Diazo compounds
6									1978.33	Aromatic system
7									1704.17	 s:
8					1558.59		1558.55	s: monomer 		
9	1412.61	1412.76	1411.57	s:  cyanate	1417.19	1411.37	1417.22	s:  cyanate	1414.46	 s:

particularly in alkali media (Destanoğlu & Gümüş-Yılmaz 2016). The photocatalytic oxidation was shown by increasing the rate of C-N=O formed in (1, 2 and 3%) SoHA-20% NCE bioplastic due to NCE addition, which low pH was caused by hydrolyzed tannin dominated by gallic acid. In zone 10, 13 and 14, both SoHA and SoHA-NCE bioplastics, ether group was formed as an indication of cross-linking process between hydroxyl group of HPS and gallic acid or amide from hydrolyzed tannin. Moreover, peaks dominated by ether and aromatic groups also found at peaks 15, 16, 18 and 19. Utilization of (1, 2 and 3%) SoHA in HPS bioplastic preparation caused SCN and C-N=O formation, while 20% NCE addition caused increasing C-N=O concentration that arguably determined bioplastic mechanical properties and antimicrobia activity.

Scanning Electron Microscopy Analysis

Micrograph resulted from the Scanning Electron Microscopy (SEM) analysis was used to study the tensile surface fracture of the eight bioplastic samples and microstructure changes caused by the addition of (1, 2 and 3%) SoHA and combination (1, 2 and 3%) SoHA-20% NCE.

The control bioplastic had homogenous surface microstructure with cracks in several parts. The microcracks formation was possibly caused by the absence of cross-linking agent in bioplastic (Ortega-toro *et al.* 2014). Addition of cross-linking agent can be detected by white spots of agglomeration on bioplastic surface made by applying addition of (2 and 3%) SoHA. The spots appeared as a side effect of uneven mixing or lack of solvent to dissolve cross-linking agent. Different to bioplastic microstucture made by using (1, 2 and 3%) SoHA, those made by using (1, 2 and 3%) SoHA-20% NCE combination had a very significantly different result compared to control, (1, 2 and 3%) SoHA and 20% NCE. Addition of 20% NCE resulted in aggregated formation on bioplastic surface, hence more flexible compared to other bioplastic samples. The flexibility was as also indicated by higher ε of bioplastic made by using (1, 2 and 3%) SoHA-20% NCE compared to the other bioplastic samples (Fig. 5).

SEM micrograph of the eight bioplastic samples showed significantly different results, with (2 and 3%) SoHA bioplastic had agglomeration as indication of cross-linking agent addition, whereas (1, 2 and 3%) SoHA bioplastic had more flexible texture from water formation because the final reaction product rendered hydrophilic property of bioplastic. NCE contains organic acid having plasticizing effect that contributes to inhibiting starch partial recrystalization (Ortega-toro *et al.* 2014).

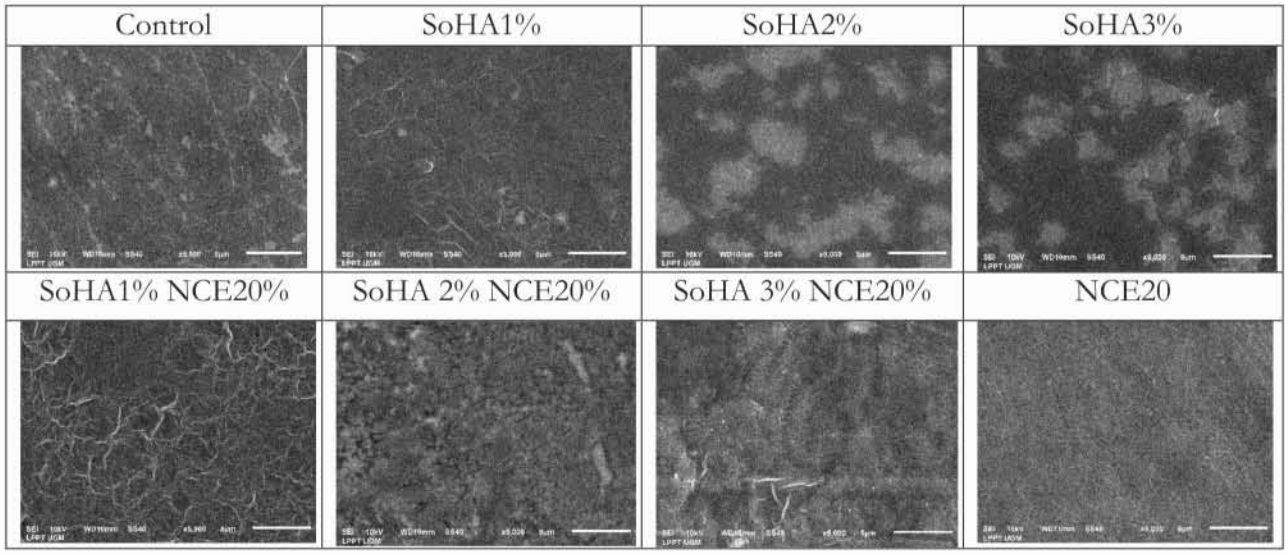


Figure 5 SEM micrographs of the control, (1, 2, and 3%) SoHA, (1, 2, and 3%) SoHA-20% NCE and 20% NCE bioplastics

Antimicrobial Analysis

Antimicrobial capabilities from control bioplastic was compared to that of (1, 2 and 3%) SoHA, (1, 2 and 3%) SoHA-20% NCE and 20% NCE bioplastics by determining the inhibition zone diameter against gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria, as well as fungi (*A. niger*).

The results showed that control and 1% SoHA had no antimicrobial activity, although the FTIR spectrum analysis showed that there is a cyanate suspected as an antimicrobial agent. The lack of antimicrobial activity was probably due to the small concentration of the cyanate. The 2 and 3% SoHA had inhibition zone indicating antibacteria activity against gram-positive bacteria (*S. aureus*) with diameter of 0.6 mm and 0.45 mm, respectively (Table 2).

The presence of antimicrobial activity was probably caused by the presence of SCN and C-N=O in the SoHA addition during bioplastic preparation, or in the SoHA addition within the HPS which generated SCN and C-N=O groups. SCN can be generated from catalysis of cyanide (Fawell *et al.* 2007). Other study conducted by (Destanoğlu & Gümüş-Yılmaz 2016) mentioned that SCN is a chronic exposure of low dose of cyanide. Besides, SCN is utilized in various industrial process, such as photofinishing, production of herbicide and insecticide, colorant, production of acrylic fiber, thio-urea production, metal separation and coating, as well as soil sterilization and corrosion inhibition (Doble & Kumar 2005). NCE addition to (1, 2 and 3%) SoHA-20% NCE bio-plastic, was able to improve antimicrobial properties against gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria as well as fungi (*A. niger*). Antimicrobial activity of (1, 2 and 3%) SoHA-20% NCE bioplastic was considered better than the control, (1, 2 and 3%) SoHA and 20% NCE, which probably due to the presence of C-N=O that was predictably increased with the increasing NCE. The existence of C-N=O is probably derived from acetanilide group found in SoHA and amide group contained in NCE. Acetanilide is the first derivative of aniline having analgesic and antipyretic properties (Khan *et al.* 2016). C-N=O concentration can be gradually increased with time, in line with the reduction of cyanide due to oxidation (Destanoğlu & Gümüş-Yılmaz 2016). The analysis results showed that (1, 2 and 3%) SoHA-20% NCE bioplastic was more sensitive against *S. aureus* than *E. coli* or *A. niger* (Table 2). This indicated that *S. aureus* was more sensitive to C-N=O in (1, 2 and 3%) SoHA-20% NCE bioplastic. Different results were obtained by 20% NCE bioplastic containing amide, toward which *E. coli* had higher sensitivity than *S. aureus*, with no antimicrobial activity against *A. niger*.

Results of this analysis also indicated that gram-positive bacteria (*S. aureus*) were more sensitive toward SCN or C-N=O in both 3% SoHA and (1, 2 and 3%) SoHA-20% NCE bioplastics compared to gram-negative bacteria (*E. coli*) and fungi (*A. niger*). However, C-N=O compounds provided antimicrobial activity for (1, 2 and 3%) SoHA-20% NCE bioplastic against *S. aureus, E. coli* and *A. niger*. On the other hand, amide content in 20% NCE showed higher sensitivity against *E. coli* than *S. aureus*, with no antimicrobial activity against fungi (*A. niger*).

Table 2 Inhibition zone obtained in the antimicrobial analysis

No	Sample	Inhibition zone against		
		E. coli	*S. aureus*	*A. niger*
1	HPS-0	0a	0a	0a
2	HPS-0-SoHA-1	0a	0a	0a
3	HPS-0-SoHA-2	0a	0a	0a
4	HPS-0-SoHA-3	0a	0.45 ± 0.26b	0a
5	HPS-20-SoHA-1	3.17 ± 0.29b	3.57 ± 0.55c	0.77 ± 0.12b
6	HPS-20-SoHA-2	2.67 ± 0.06c	3.23 ± 0.31c	0.80 ± 0.00b
7	HPS-20-SoHA-3	2.40 ± 0.17d	2.97 ± 0.42d	0.57 ± 0.06c
8	HPS-20	5.07 ± 0.20e	4.32 ± 0.25e	0a

CONCLUSION

Addition of (1, 2, and 3%) SoHA as cross-linking agent and combination of (1, 2, and 3%) SoHA-20% NCE had significant effects on mechanical properties and antimicrobial activity of HPS bioplastic. SoHA at 2 and 3% in HPS bioplastic improved mechanical properties in form of σ and Y increase while reducing Ɛ, WS and WVP. The reduction was caused by the presence of SCN and C-N=O which probably derived from acetanilide group found in SoHA causing antimicrobial activity against gram-positive bacteria (*S. aureus*). On the contrary, combination of (1, 2 and 3%) SoHA-20% NCE degraded mechanical properties by reducing σ and Y followed by Ɛ, WS and WVP decrease, which probably due to water formation from reactions involving HPS, SoHA, and NCE. However, antimicrobial analysis indicated sensitivity against gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria as well as fungi (*A. niger*) which arguably caused by C-N=O formation increase in bioplastic, originating from acetanilide group in SoHA and amide group in NCE.

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REFERENCE

Aguirre A, Borneo R, Leon AE. 2013. Antimicrobial, mechanical and barrier properties of triticale protein films incorporated with oregano essential oil. Food Biosci 1:2-9. https://doi.org/10.1016/j.fbio.2012.12.001

Bilal M, Niazi K, Broekhuis AA. 2015. Surface photo-crosslinking of plasticized thermoplastic starch films. Eur Polym J 64:229-43. https://doi.org/10.1016/ j.eurpolymj. 2015.01.027

Canisag H. 2015. Bio-cross-linking of starch films with oxidized sucrose. textiles, merchandising and fashion design. Dissertations, Thesis, & Student Research 6. The Faculty of The Graduate College at the University of Nebraska.

Chana-Thaworn J, Chanthachum S, Wittaya T. 2011. Properties and antimicrobial activity of edible films incorporated with kiam wood (*Cotyleobium lanceotatum*) extract. Food Sci Technol 44(1):284-92. https://doi.org/10.1016/j.lwt.2010.06.020

Chandler JD, Day BJ. 2012. Thiocyanate: A potentially useful therapeutic agent with host defense and antioxidant properties. Biochem Pharmacol 84(11):1381-7. https://doi.org/10.1016/j.bcp.2012.07.029

Chanwitheesuk A, Teerawutgulrag A, Kilburn JD, Rakariyatham N. 2007. Antimicrobial gallic acid from *Caesalpinia mimosoides* Lamk. Food Chem 100(3):1044-8. https://doi.org/10.1016/j.foodchem.2005.11.008

Contipro. 2018. Speciality hyaluronan chemicals, contipro. Available at: http://www.phadia.com/ Global/A Document Library/Product Catalogues/Product-Catalog-2018.pdf.

Destanoğlu O, Gümüş-Yılmaz G. 2016. Determination of cyanide, thiocyanate, cyanate, hexavalent chromium, and metal cyanide complexes in various mixtures by ion chromatography with conductivity detection. J Liq Chromatogr Relat 39(9):465-74. https://doi.org/10.1080/ 10826076.2016.1192044

Djajadisastra J, Dzuhro ZS, Sutriyo. 2014. Pengaruh natrium hialuronat terhadap penetrasi kofein sebagai antiselulit dalam sediaan hidrogel, hidroalkoholik gel, dan emulsi gel. Pharm Sci Res 1(1):46-63.

Doble M, Kumar A. 2005. 'Cyanide Waste', in biotreatment of industrial effluents. E-book. Elsevier Inc. pp.177-82. https://doi.org/ 10.1016/b978-075067838-4/50017-8.

Espinel RA, Flores SK, Gerschenson LN. 2014. Biopolymeric antimicrobial films: Study of the influence of hydroxypropyl methylcellulose, tapioca starch and glycerol contents on physical properties. Mater Sci Eng C 36:108-17. https://doi.org/10.1016/j.msec. 2013.11.043

Fawell JK, Cotruvo J, Giddings M, Jackson P, Magara Y, Ohanian E. 2007. Cyanide in drinking water. Geneva (CH): World Health Organization. p.1-26.

Frost K, Johansson E, Shanks R, Goto S, Kirwan GM. 2013. Thermoplastic starch films: DOE and O2PLS methodology for optimization and increased understanding of polymer processing. Polym Test 32(2):343-52. https://doi.org/10.1016/j.polymertesting.2012.10.005

Gui-jie M, Peng W, Xiang-sheng M, Xing Z, Tong Z. 2006. Crosslinking of corn starch with sodium trimetaphosphate in solid state by microwave irradiation. J Appl Polym Sci 102: 5854-60. https://doi.org/10.1002/app.24947

Gutiérrez TJ, Morales NJ, Tapia MS, Pérez E, Famá L. 2015. Corn Starch 80:20 "Waxy": Regular, "Native" and Phosphated, as bio-matrixes for

edible films. Procedia Materials Science 8: 304-10. https://doi.org/10.1016/j.mspro.2015.04.077

Hassannia-kolaee M, Khodaiyan F, Pourahmad R, Shahabi-Ghahfarrokhi I. 2016. Development of ecofriendly bionanocomposite: Whey protein isolate/pullulan films with nano-SiO_2. Int J Biol Macromol 86:139-44. https://doi.org/ 10.1016/ j.ijbiomac.2016.01.032

Khan MF, Razan R, Rashid RB, Tahia F, Rashid MA. 2016. Computational study of geometry, IR spectrum and molecular properties of acetanilide. Bangladesh J Pharmacol 19(2):170-8. https://doi.org/10.3329/bpj.v19i2.29276.

Kumari M, Mahajan H, Joshi R, Gupta M. 2017. Development and structural characterization of edible films for improving fruit quality. Food Packag Shelf Life 12:42-50. https://doi.org/ 10.1016/j.fpsl.2017.02.003

Leceta I, Guerrero P, Ibarburu I, Dueñas MT, Caba KD. 2013. Characterization and antimicrobial analysis of chitosan-based films. J Food Eng 116(4):889-99. https://doi.org/10.1016/ j.jfoodeng.2013.01.022

Manoi K, Rizvi SSH. 2010. Physicochemical characteristics of phosphorylated cross-linked starch produced by reactive supercritical fluid extrusion. Carbohydr Polym 81:687-94. https://doi.org/10.1016/j.carbpol.2010.03.042

Ortega-toro R, Jiménez A, Talens P, Chiralt A. 2014. Properties of starch–hydroxypropyl methyl-cellulose based films obtained by compression molding. Carbohydr Polym 109:155-65. https:// doi.org/10.1016/j.carbpol.2014.03.059

Rasheed F, Hedenqvist MS, Kuktaite R, Plivelic TS, Gällstedt M, Johansson E. 2015. Mild gluten separation: A non-destructive approach to fine tune structure and mechanical behavior of wheat gluten films. Ind Crops Prod 73:90-8. https://doi.org/10.1016/j.indcrop.2015.04.007

Ricci A, Olejar KJ, Parpinello GP, Kilmartin PA, Versari A. 2015. Application of Fourier transform infrared (FTIR) spectroscopy in the characterization of tannins. Appl Spectrosc Rev 50(5):407-42. https://doi.org/10.1080/05704928.2014.1000461

Ruckmani K, Shaikh SZ, Khalil P, Muneera MS, Thusleem OA. 2013. Determination of sodium hyaluronate in pharmaceutical formulations by HPLC-UV. J. Pharm. Anal. 3(5):324-9. https://doi: 10.1016/j.jpha.2013.02.001.

Samsudin H, Hani NM. 2019. 'Use of starch in food packaging'. In Starch-based materials in food packaging. Academic Press. p.229-56. https://doi: 10.1016/B978-0-12-809439-6.00008-X.

Shah U, Naqash F, Gani A, Masoodi FA. 2016. Art and science behind modified starch edible Films and coatings: A review. Compr Rev Food Sci Food Saf 15:568-80. https://doi.org/10.1111/1541-4337. 12197

Simeonova FP, Fishbein L. 2004. Hydrogen cyanide and cyanides: Human health aspects. Geneva (CH): World Health Organization.

Spec-Chem Ind. 2014. Sodium Hyaluronate. Spec-Chem, China.

Umiyati R, Millati R, Ariyanto T, Hidayat C. 2019. Physical and antimicrobial properties of hydroxypropyl starch bio-plastics incorporated with nyamplung (*Calophyllum inophyllum*) cake extract as an environmentally friendly food packaging. E3S Web of Conferences 125, 07011. International Conference on Energy, Environment, Epidemiology and Information, Universitas Diponegoro.

Woggum T, Sirivongpaisal P, Wittaya T. 2015. Characteristics and properties of hydroxy-propylated rice starch based biodegradable films. Food Hydrocoll 50: 54-64. https://doi.org/ 10.1016/ j.foodhyd.2015.04.010

Wypych G. 2017. Typical methods of quality Control of plasticizers. A handbook of plasticizers (3rd edition). Toronto (CA): ChemTec Publishing. pp.85-109. https://doi.org/10.1016/B978-1-895198-97-3.50003-2

Xuejun C, Xiaobin Z, Zou, Y. 2015. Cross-linked sodium hyaluronate gel for tissue filler for plastic surgery and preparation method Thereof 1(19): 1-4. United States: 14/432 (a patent).

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LITTERFALL, LITTER DECOMPOSITION AND NUTRIENT RETURN OF REHABILITATED MINING AREAS AND NATURAL FOREST IN PHANGNGA FORESTRY RESEARCH STATION, SOUTHERN THAILAND

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ABSTRACT

Litterfall and litter decomposition play important roles in the maintenance of nutrient cycling and rehabilitation of degraded lands. Litterfall, litter decomposition and nutrient return were investigated in a 27-year-old *Acacia mangium* plantation on sandy and clay sites, and in a mixed plantation at the Phangnga Forestry Research Station, Phangnga Province, Thailand. Additionally, secondary and primary forests were investigated and compared with the values obtained from the *Acacia mangium* and the mixed plantations. The results indicated that litter production in *A. mangium* plantation on sandy and clay sites, and in mixed plantations (15.47, 11.68 and 7.89 t/ha/yr, respectively) was higher than that in the secondary and primary forests (6.34 and 6.92 t/ha/yr, respectively). The rate of litter decomposition was the greatest in the secondary forest (3.01/yr) and the lowest occurred in the primary forest (1.15/yr). The decomposition rate of the mixed leaf litter between native trees and *A. mangium* in plantations was higher than that of only *A mangium* leaf, except in the mixed plantations. A high initial nitrogen concentration in *A. mangium* could accelerate litter decomposition and improve litter quality in the mixed litter. In addition, the nutrient return in plantations was higher than that in the secondary and primary forests, especially for N. Increased litter production, high decomposition rate and nutrient return from *A. mangium* plantation had important roles in nutrient cycling, suggesting that a mixed plantation consisting of *A. mangium* and native trees should be considered for the reclamation of mining land.

Keywords: litter decomposition, litterfall, mining rehabilitation, nutrient return, tropical forest

INTRODUCTION

Mining operations have been undertaken on a global scale as the raw minerals obtained play an important role in economic and infrastructure development. A high level of mineral production has been reported in Asian countries (Reichl *et al.* 2017). The ill-effects of mining cause severe impacts on the ecosystem, including soil degradation, water and air pollution, and loss of wildlife habitat. The vegetation richness and soil properties are destroyed by mining activities, resulting in the degradation of the ecosystem (Bell & Donnelly 2006; Tripathi *et al.* 2016). Improving environmental conditions in mining areas using natural methods takes a long time (Oktavia *et al.* 2015). Barriers to natural regeneration include environmental factors such as water, soil pH and soil properties (Thaiutsa & Rungruangsilp 1990; Tripathi *et al.* 2016).

Forest plantations have been established on degraded lands to improve the soil properties and ecosystem function (Singh *et al.* 2004; Zhang *et al.* 2014; Oktavia *et al.* 2015). Rehabilitation in such areas by planting nitrogen-fixing trees has been recommended and they have been planted on many sites as

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they grow well and have a high aboveground biomass and litter production (Dutta & Agrawal 2003; Singh *et al.* 2004). *Acacia mangium* is the most preferred species for reclamation efforts in mining land (Martpalakorn 1990; Oktavia *et al.* 2015) and other degraded lands (Kamo *et al.* 2008). Soil properties and microclimate under such plantations are improved, resulting in the accelerated natural regeneration of native forest species (Parrotta 1999; Inagaki *et al.* 2010).

Litter production and decomposition play important roles in nutrient cycling and the improvement of soil fertility in terrestrial ecosystems (Goma-Tchimbakala & Bernhard-Reversat 2006; Paudel *et al.* 2015). On degraded lands, litterfall and litter decomposition are keys to restoring the ecology and soil properties because the litter is a nutrient source (Lugo 1992; Parsons & Congdon 2008). Nutrient contents of plants are closely related to tree species, especially nitrogen which is abundant in nitrogen-fixing trees (Parrotta 1999; Singh *et al.* 2004). Tree composition and forest type are the main factors influencing the quality of the litter produced (Parrotta 1999; Zhou *et al.* 2006; Tang *et al.* 2010; Paudel *et al.* 2015). In addition, climatic factors can significantly affect litterfall (Zhou *et al.* 2006; Scherer-Lorenzen *et al.* 2007; Triadiati *et al.* 2011). Litter fractions that fall onto the forest floor are decomposed by organisms living in the soil, and nutrients are subsequently released to the forest floor, resulting in the improved soil properties and a better forest community, which maintain forest function (Parrotta 1999). Litter quality and microclimate are related to the litter decomposed by soil microbes (Parsons & Congdon 2008; Cizungu *et al.* 2014; Zhong *et al.* 2017). Furthermore, litter decomposition varies depending on the tree species, successional stage, and forest type (Lugo 1992; Parsons & Congdon 2008; Tang *et al.* 2010).

The efficiency of plantations in degraded land rehabilitation not only builds productivity and improves forest structure but also increases forest function such as nutrient cycling (Lugo 1992; Parrotta 1999). A high litter decomposition rate and nutrient return encouraged natural succession and increased soil nutrients (Lugo 1992; Celentano *et al.* 2011), which reduced the time required for the restoration process. Therefore, the objective of the current study was to evaluate the potential of *A. mangium* plantation for mining rehabilitation, focusing on litter production, decomposition and nutrient return in comparison to secondary and primary forests. An effort has been made to compare the rehabilitated plantation area to secondary and primary forests.

MATERIALS AND METHODS

Study Sites

This study was carried out in an abandoned tin mining area, in the Phangnga Forestry Research Station, Phangnga Province, Southern Thailand (8°46′5″N, 98°16′7″E) (Fig. 1). Exotic trees, such as *Acacia mangium* and *Eucalyptus camaldulensis* were commonly planted for tin mining reclamation in Thailand. The mining was operated by the gravel pumping method. Landform after mining was divided into clay, sand, and gravel areas. Soil nutrients were observed to be very low (Thaiutsa & Rungruansilp 1990). The experimental plots were established in a 27-year-old *A. mangium* plantation, planted in a sandy soil area (AMS), in a clay soil area (AMC), and mixed plantation (MP). The planted trees in MP consisted of *A. mangium*, *Eucalyptus camaldulensis* and *Dipterocarpus alatus*.

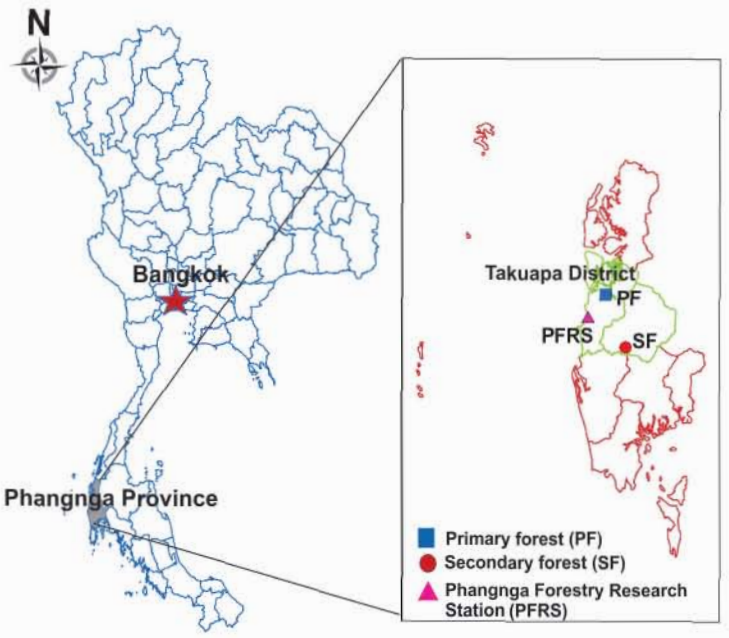

Figure 1 Location of study sites in Phangnga Province, Southern Thailand

In addition, the experimental plots were established in a secondary forest (SF, located at 8°39′28″ N, 98°24′35″ E) and a primary forest (PF, located at 8°51′11″ N, 98°20′5″ E) as reference sites. The SF plot was in an approximately 30-year-old site abandoned after shifting cultivation. The PF plot was classified as a low tropical rainforest. Dominant native trees in the 27-year-old plantations and the secondary forest were pioneer tree species including *Carallia brachiata*, *Aporosa planchoniana*, *Bridelia tomentosa*, *Vitex pinnata*, *Microcos paniculata* and *Eurya acuminata*. Meanwhile, dominant trees in PF were *Swintonia schwenckii*, *Dipterocarpus kerrii*, *Mesua ferrea*, *Hopea griffithii* and *Gluta elegans*. Tree composition and dominant trees in AMS, AMC, MP, SF and PF were reported by Wongprom *et al.* (2020).

Climatic conditions during the study were recorded at the climate station in the Takuapa District. The rainfall was 3,260.10 mm with the rainy season occurring from April to November and the dry season happening from December to March. The mean relative humidity was 83% and the mean temperature was 27.58 °C. Data on rainfall, mean temperature and relative moisture recorded during the study are shown in Figure 2.

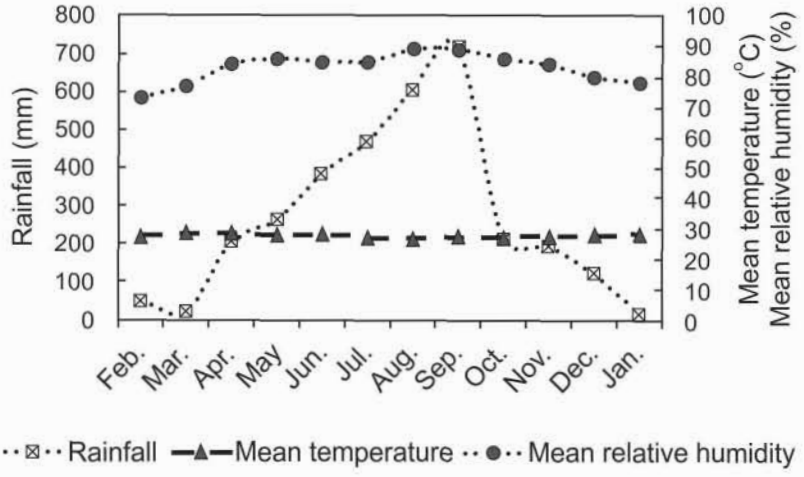

Figure 2 Climatic conditions at Takuapa District, Phangnga Province during the study

Litter Production

Three experimental plots (each 40 x 40 m) were established in each AMS, AMC, MP, SF, and PF for estimating the amount of litterfall production. Five litter traps (each 1 x 1 m) with a 2 mm mesh size were placed 1 m above the ground in each plot. Litterfall in the traps was collected monthly for 1 year. Litterfall samples were sorted into leaf, branches, reproductive parts and miscellaneous. *A. mangium* litter was separated from that of the other tree species. All samples were oven-dried at 80 °C for 48 h until reaching constant weight. The samples (leaf, branches, reproductive parts and miscellaneous) were analyzed during both the rainy and dry seasons. The organic debris obtained from each site was analyzed as a single sample, as it was difficult to identify the components as they were very small and had a similar texture.

Litter Decomposition and Nutrient Return

Nylon bags (50 x 50 cm) with a 2 mm mesh size were used for investigating litter decomposition. Leaf having the top five Importance Value Index (IVI) trees in AMS, AMC, MP and SF, and the top seven IVI trees of PF were selected to investigate litter decomposition. Litter decomposition was divided into two classes: 1) mixed leaf litter in AMS, AMC, MP, SF and PF; and 2) pure *A. mangium* leaf litter in AMS, AMC, and MP. In each case, 30 g samples of air-dried leaf litter were filled into the nylon bags according to the IVI ratio of the dominant trees for each site. The IVI of trees in AMS, AMC, MP, SF and PF was reported by Wongprom *et al.* (2020). The litter water content was determined by first, oven-drying subsamples of air-dried litter at 80 °C for 48 h to obtain the initial dry mass. Twelve litter bags from each treatment with three replications were randomly placed in each plot. Three samples of both mixed leaf litter and pure *A. mangium* leaf litter were retrieved monthly. The leaf litter remaining in each bag was brushed to remove any soil particles and roots. The remaining litter was oven-dried at 80 °C for 48 h until a constant weight was recorded.

The annual litter decomposition rate (k) was calculated according to the negative exponential decay model (Olson 1963) given as $k = \ln (X/X_0)/t$, where X_0 is the initial dry weight, X is the dry weight remaining at the end of the study, and t is the time period in years. Nutrient return (N, P, K, Ca, and Mg) to the forest floor (kg/ha) was estimated by multiplying the amount of litter production by the nutrient concentration of litter for each site.

Chemical Analysis of Litter

Nutrient concentrations of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) of leaf, branches, reproductive parts and miscellaneous obtained from the study sites were determined during the rainy (April to November) and dry (December to March) seasons. Samples of each litter type in the rainy and dry seasons were mixed and analyzed. The concentration of N was measured based on dry combustion using a CNHS analyzer, while the concentrations of P, K, Ca, and Mg were determined by wet washing with NHO_3-$HClO_4$ acid (HNO_3: $HClO_4$; 5: 2). The concentration of P was analyzed using the vanadomolybdate yellow color method with a spectrometer at a wavelength of 440 nm, while the concentrations K, Ca, and Mg were analyzed using atomic absorption spectrometry.

Data Analysis

Nutrient concentrations of each litter sample during the rainy and dry seasons were averaged to calculate the amount of nutrients return. The annual litter production, nutrient return and litter decomposition rate among sites were analyzed using a one-way ANOVA and the means were compared with the Tukey HSD test at a 5% probability level.

RESULTS AND DISCUSSION

Litter Production

The annual litter production was significantly different among sites ($P < 0.05$). AMS had the highest litterfall followed by AMC, MP, PF, and SF (Table 1).

Table 1 Litter production (leaf, branches, reproductive parts and miscellaneous) (t/ha/yr) of *A. mangium* (AM) and native trees and planted trees (NPT) in the 27-year-old *A. mangium* plantation in sandy soil area (AMS), clay soil area (AMC), mixed plantation (MP), secondary forest (SF), and primary forest (PF)

Site	Leaf		Branches		Reproductive parts		Miscellaneous	Total
	AM	NPT	AM	NPT	AM	NPT		
AMS	5.90^{a}	2.85^{b}	0.74	1.67	2.86^{a}	0.57	0.88^{a}	15.47^{a}
AMC	3.56^{ab}	3.83^{ab}	0.56	1.20	1.60^{b}	0.50	0.43^{b}	11.68^{b}
MP	1.52^{b}	2.86^{b}	0.59	1.06	0.84^{b}	0.51	0.51^{ab}	7.89^{c}
SF	-	4.12^{ab}	-	0.76	-	0.74	0.72^{ab}	6.34^{c}
PF	-	4.72^{a}	-	0.89	-	0.72	0.59^{ab}	6.92^{c}
F value	11.59**	8.03**	1.83^{ns}	0.87^{ns}	16.55**	2.02^{ns}	4.58*	27.21**

Notes: * = significant difference; ** = very significant difference; ns = non-significant difference; a - c = different superscripts in the same column indicate significant differences at $P < 0.05$.

Litter production peaked in the dry season (December to March) (Fig. 3), as a response to the water stress, which was similar to that of other tropical forests (Triadiati *et al.* 2011; Cizungu *et al.* 2014). Leaf component was the main litter falling onto the forest floor in AMS, AMC, MP, SF and PF, and made up around 54 to 62% of the total litter. However, the amount of reproductive parts in AMS and AMC was relatively high compared to branches, especially the reproductive parts of *A. mangium* as it fell almost all year. A large amount of litter from *A. mangium* in AMS was resulted from the high tree density and crown cover, while *A. mangium* in AMC and MP had a low crown cover due to the high mortality rate. Litters from other trees in AMS, AMC, and MP came from native tree species. Planted trees in MP such as *D. alatus* and *E. camaldulensis* were also a main source of litter. Leaf litter is a significant contributor of annual primary production and nutrient capital in a terrestrial ecosystem (Cizungu *et al.* 2014; Paudel *et al.* 2015).

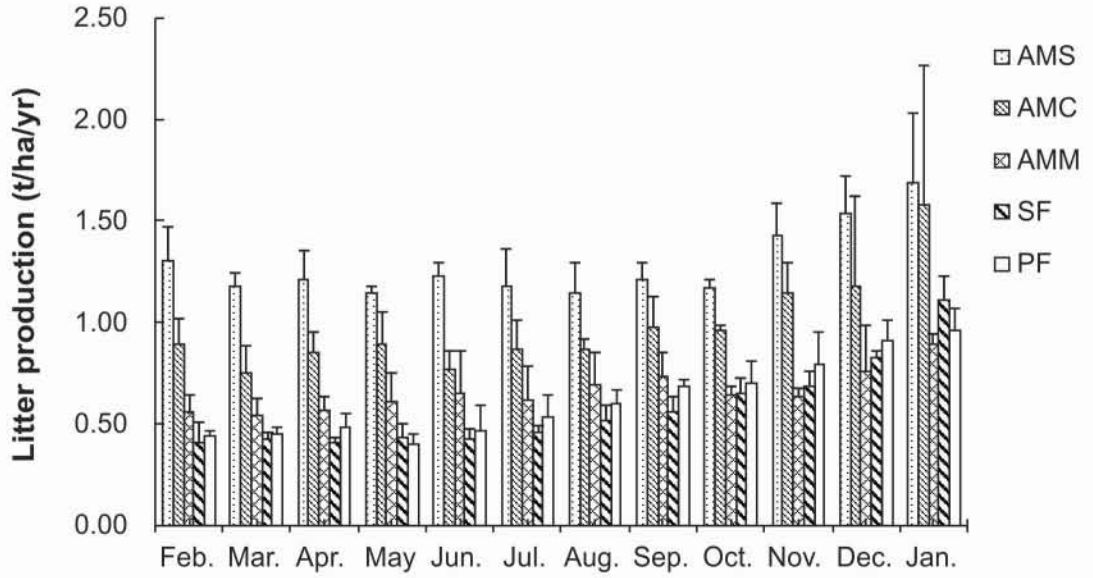

Figure 3 Total monthly litter production (t/ha/yr) of the 27-year-old *A. mangium* plantation in sandy soil area (AMS), clay soil area (AMC), mixed plantation (MP), secondary forest (SF), and primary forest (PF)

This study showed that the amount of litter production in AMS and AMC was higher than that in both SF and PF. A forest plantation with fast-growing trees is highly effective at building litter production compared to a natural forest (Goma-Tchimbakala & Bernhard-Reversat 2006) and secondary forest (Lugo 1992; Kamo *et al.* 2008). Post mining reclamation should focus on increasing the aboveground biomass and litter production to improve the ecosystem. *A. mangium* is one species recommended for degraded land restoration. Acacia grows well and has high aboveground biomass compared to native trees (Martpalakorn 1990; Kamo *et al.* 2008). However, the total litter production in MP was similar to that in PF and SF, although *A. mangium* had less litter production. The canopy cover of *A. mangium* can promote a high litterfall. Fast nitrogen-fixing tree plays important role in the early stage by rapid growth to create a canopy in order to suppress the weeds and shrubs that could obstruct the establishment of native tree understory vegetation and to increase nitrogen availability (Ingaki *et al.* 2010; Lanuza *et al.* 2018). However, the litterfall in AMS, AMC and MP was not only sourced from planted trees but also native trees through natural succession. Old plantations have high tree diversity and richness (Lugo 1992; Koonkhunthod *et al.* 2007), as litterfall is contributed to by native trees. Native tree species are usually pioneer trees having high growth rates (Chazdon 2014; Chen *et al.* 2017) and their litter production increases with succession age (Zhou *et al.* 2006; Feng *et al.* 2019). Litterfall production plays an important role in forest functions such as carbon and nutrient cycling (Moura *et al.* 2016; Paudel *et al.* 2015; Lanuza *et al.* 2018) because litter is a source of nutrient pools, which releases nutrient to the forest floor via litter decomposer activities (Lugo 1992; Fisher & Binkley 2000), suggesting that *A. mangium* should be recommended for reclamation in a mining area due to the capability of *A. mangium* in bringing out high litter production.

Litter production in the SF and PF study sites was not different, resulting from pioneer trees producing litter in natural succession. Forest structure and tree composition of succession forests affected litter biomass (Lanuza *et al.* 2018). Long-lived pioneer trees and high tree diversity were observed in SF. The litter production in the SF and PF study sites was similar to that in many evergreen forests in Thailand (6.42 to 7.85 t/ha/yr) (Bunyavejchewin 2001; Glumphabutr *et al.* 2007). However, litter production in our study was lower than that in other tropical forests (8.30 - 13.67 t/ha/yr) (Anderson *et al.* 1983; Tang *et al.* 2010; Triadiati *et al.* 2011; Paudel *et al.* 2015).

Litter Decomposition

Litter decomposition of pure litter and mixed litter was significantly different among treatments ($P < 0.05$). The litter decomposition rate (k) ranged between 1.15 and 3.01/yr, which was classified as medium to high levels. The decomposition rate in SF was the highest, indicating litters were rapid litter decay, but not different in AMC (2.91/yr) (Table 2).

The dominant trees in SF and AMC were pioneer tree species and were of similar tree composition (Wongprom *et al.* 2020), having thin and flexible leaves without a prominent skeleton, and so could decay easily. On the other hand, a low rate of decomposition was found in climax forest trees due to their thick, tough leaf and prominent midribs and veins. The leaf morphology of trees (leaf thickness, roughness, toughness) significantly affects the rate of litter decomposition (Liao *et al.* 2006; Cizungu *et al.* 2014). In addition, the chemical properties of leaf litter, including high N, N/P, low C/N and low lignin, are generally positive contributors to decomposition (Xuluc-Tolosa *et al.* 2003; Liao *et al.* 2006; Cizungu *et al.* 2014; Rai *et al.* 2016). Similarly, leaf litter in SF, AMS, AMC, and MP had a high nitrogen content, which could explain their faster rate of decomposition. Leaf litter in AMS, AMC, and MP was rich in nitrogen due to the litter produced by *A. mangium*. In addition, leaves of dominant pioneer trees in SF, AMS AMC and MP have thin and less rigid leaves leading to a high decomposition rate compared to PF. However, the litter decomposition of PF was of moderate level (1.15/yr), which was similar to other climax tropical forests (Hättenschwiler *et al.* 2011).

Table 2 Decomposition rate of pure *A. mangium* and the mixed leaf in the 27-year-old *A. mangium* plantation in sandy soil area (AMS), clay soil area (AMC), mixed plantation (MP), secondary forest (SF), and primary forest (PF)

Site	Type of leaf	*k* (constant)
AMS	Pure *A. mangium* leaf	1.56^{bc}
	Mixed leaf	1.64^{bc}
AMC	Pure *A. mangium* leaf	1.88^{bc}
	Mixed leaf	2.91^{a}
MP	Pure *A. mangium* leaf	2.05^{b}
	Mixed leaf	1.87^{bc}
SF	Mixed leaf	3.01^{a}
PF	Mixed leaf	1.15^{cd}
F value		7.905*

Notes: * = significant difference; a - d = different superscripts in the same column indicate significant differences at $P < 0.05$.

Climate conditions and soil microbials significantly affect litter decomposition (Berg & McClaugherty 2008). This study indicated that rainfall and relative humidity in the study sites were of similar conditions. However, soil microbials could be different because soil properties in AMS, AMC, and MP were of poor soil compared to that in SF and PF, especially in terms of total N and OM (Wongprom *et al.* 2020), leading to low diversity and abundance of soil microbials (Zhang *et al.* 2016). Soil nutrients, N and P and Organic Matter (OM) are significantly correlated with soil microbials (Zhang *et al.* 2016; Ngugi *et al.* 2020). Low decomposition rate of litter was found in the PF study site. Microclimate conditions may slightly affect litter decomposition. Litter quality of the restored forest may significantly determine litter decomposition. Meanwhile, litter decomposition rate changes depending on successional stage and tree composition (Moura *et al.* 2016; Lanaza *et al.* 2018). A study conducted by Xuluc-Tolosa *et al.* (2003) indicated that pioneer trees in the early succession phase has a higher decay rate than tree species in the late succession phase because pioneer trees have a high initial N concentration and low C/N. Pioneer tree species have high nutrient resorption efficiency and high leaf nutritional quality, especially N concentration (Gomes & Luizão 2012). Our study showed that a litter mixture of *A. mangium* and native trees generally resulted in a faster decay than the decay of PF litter. This result could be affected by synergistic effects of mixed litter with high litter quality and a high initial N concentration. Mass loss is positively correlated with initial N concentration (Liu *et al.* 2016). Thus, poor litter quality is improved. Mixing litters from various trees accelerate the mass loss, enhance the nutrient release and nutrient cycling (Liu *et al.* 2016; Trogisch *et al.* 2016; Cizungu *et al.* 2016) and different litter components may change the litter chemical components and the decomposer community (Gartner & Cardon 2004).

Nutrient Concentration in Litter and Nutrient Return

Nutrient concentration in litter produced by *A. mangium* in this study varied among components (leaf, branches and reproductive parts). However, the N concentration was greater than that of P, K, Ca, and Mg (Fig. 4). Nutrient concentration in the leaf and branches litters of *A. mangium* in all sites followed the order of N>Ca>K>Mg>P, except for the reproductive parts, where the K concentration was higher than that of Ca.

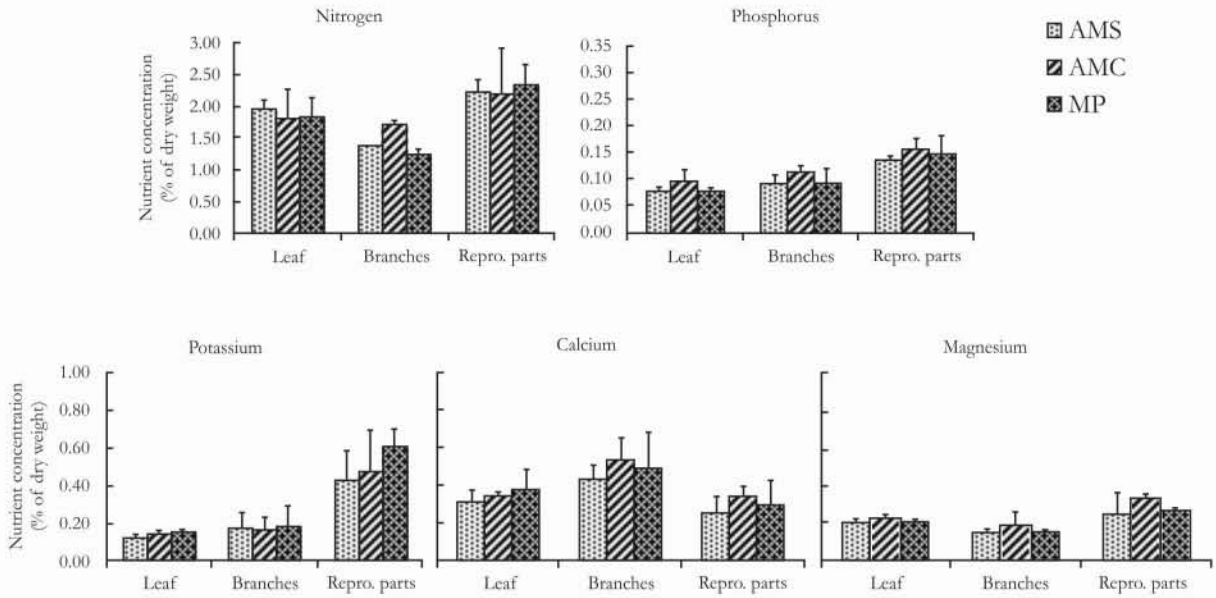

Figure 4 Nutrient concentration of pure *A. mangium* leaf, branches and reproductive parts of the 27-year-old *A. mangium* plantation in sandy soil area (AMS), clay soil area (AMC) and mixed plantation (MP)

N concentration in the litter of other trees (native tree species in AMS, AMC, MP, SF and PF, and planted trees in MP) was the highest for all litter components, and was especially high in the litter miscellaneous component. The P concentration was relatively high in the miscellaneous and reproductive parts components, while the concentrations were similar for K, Ca and Mg in the leaf, branches, reproductive parts and miscellaneous components. The nutrient concentration of leaf, branches, reproductive parts and miscellaneous for the other tree species in AMS, AMC, MP, SF, and PF are shown in Figure 5.

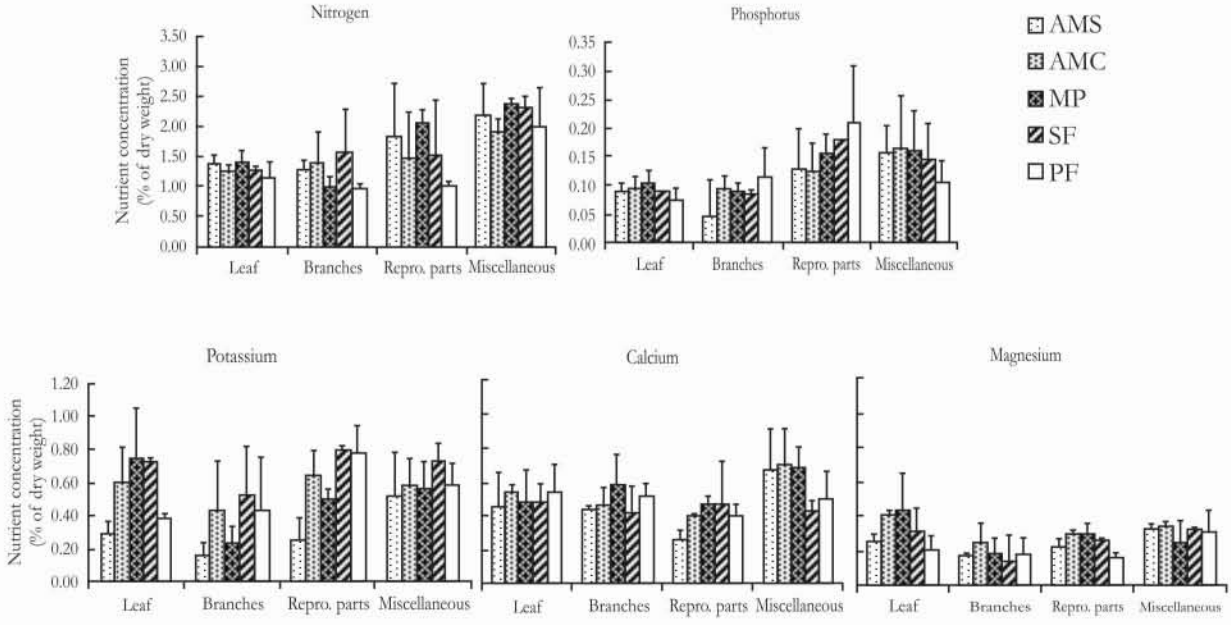

Figure 5 Nutrient concentration of mixed leaf, branches, reproductive parts and miscellaneous litter of native tree species in the 27-year-old *A. mangium* plantation in sandy soil area (AMS), clay soil area (AMC), secondary forest (SF), and primary forest (PF) and planted trees and native tree species in mixed plantation (MP)

The nutrient return of N, P, K, Ca and Mg from litters to forest floor was significantly different among sites. N contributed the highest nutrient return to the forest floor in all sites. The large amounts of N, P, K, Ca and Mg returned in AMS, AMC, and MP were mostly from the litter of the planted trees, especially *A. mangium* (Table 3).

A. mangium is the dominant tree with a large size and crown cover, although the tree density is low, particularly in the AMC and MP sites (Wongprom *et al.* 2020). Litters from planted trees in a plantation are still the main nutrient source to the forest floor, although the vegetation composition are shifted after restoration. Leaf litter was the main source of nutrient return in all sites. However, nutrient return from miscellaneous and reproductive parts of *A. mangium* was relatively high. The annual nutrient return of AMS, AMC and PF followed a pattern of N>Ca>K>Mg>P, while that of MP and SF followed a pattern of N>K>Ca>Mg>P (Table 4). The annual nutrient return of N, P, Ca and Mg to forest floor in AMS and AMC was significantly higher than that in SF and PF, especially for the nitrogen. Meanwhile, the annual nutrient return of N, P, Ca, and Mg in SF was similar to that in PF.

Forest community on a restored site and in a secondary forest can accelerate litter decomposition and nutrient cycling because the litter quality is improved. Nutrients returned to the forest floor can promote natural regeneration and tree growth, resulting in a complex forest structure in the long term, especially as N is a significant nutrient in developing a forest community and establishing seedlings and saplings during mining restoration and natural succession in a degraded land (Zhao *et al.* 2013; Lei *et al.* 2015). Fast litter decomposition of restored sites resulted in high nutrient depositions, especially N.

In the current study, N was the major nutrient return in AMS, AMC and MP, and was significantly higher than that in SF and PF. The high N return may be resulted from *A. mangium* due to the Acacia being a nitrogen-fixing tree. Moreover, the return of P, K, Ca and Mg to the soil of the rehabilitated sites was greater than that in PF. Soil nutrients of *A. mangium* plantation in an abandoned mining area are higher compared to that in an abandoned mining area without the *A. mangium* plantation, especially in terms of N and soil organic matter (Wongprom *et al.* 2020). Therefore, the study result indicated that *A. mangium* improves soil chemical properties through nutrient cycling processes. N flux of nitrogen-fixing tree plays an important role in the early succession (Moura *et al.* 2016).

Table 3 Nutrient return (kg/ha/yr) of *A. mangium* (AM) litter and native trees and planted trees (NPT) litter in the 27-year-old *A. mangium* plantation in sandy soil area (AMS), clay soil area (AMC), and mixed plantation (MP)

Site	N		P		K		Ca		Mg	
	AM	NPT	AM	NPT	AM	NPT	AM	NPT	AM	NPT
AMS	18.96^{a}	7.12	0.94^{a}	0.49	2.07^{a}	1.48^{b}	2.92^{a}	2.14^{ab}	1.91^{a}	1.20^{b}
AMC	10.97^{a}	6.52	0.68^{a}	0.52	1.37^{a}	2.93^{a}	2.08^{ab}	2.64^{a}	1.41^{a}	1.94^{a}
MP	5.47^{b}	6.42	0.30^{b}	0.49	0.85^{b}	2.73^{a}	1.11^{b}	1.91^{b}	0.62^{b}	1.56^{ab}
F value	26.37^{**}	1.09^{ns}	18.39^{**}	0.37^{ns}	14.95^{**}	29.01^{**}	14.48^{**}	6.19^{*}	15.66^{**}	12.36^{**}

Notes: * = significant difference; ** = very significant difference; ns = non-significant difference; a - b = different superscripts in the same column indicate significant differences at $P < 0.05$.

Table 4 Nutrient return (kg/ha/yr) of litter to the forest floor in the 27-year-old *A. mangium* plantation in sandy soil area (AMS), clay soil area (AMC), mixed plantation (MP), secondary forest (SF), and primary forest (PF)

Site	N	P	K	Ca	Mg
AMS	26.09^{a}	1.43^{a}	3.56^{b}	5.06^{a}	3.12^{a}
AMC	17.49^{b}	1.19^{a}	4.30^{a}	4.72^{a}	3.36^{a}
MP	11.89^{c}	0.79^{b}	3.58^{b}	3.03^{b}	2.18^{bc}
SF	8.26^{c}	0.63^{b}	4.21^{ab}	2.72^{b}	1.74^{bc}
PF	7.47^{c}	0.61^{b}	2.77^{c}	3.20^{b}	1.28^{c}
F value	53.60^{**}	33.78^{**}	15.87^{**}	22.75^{**}	34.63^{**}

Notes: ** = significant difference; a - c = different superscripts in the same column indicate significant differences at $P < 0.05$.

Fast-growing tree plantations are commonly planted for wood production. However, they are also planted for forest restoration in degraded lands. High litter production and litterfall are significant parts in restoration processes (Parrotta 1999). The N nutrient returned to the forest floor from nitrogen-fixing tree plantations was significantly higher than that from a non-nitrogen-fixing tree plantations and for other nutrients (Bernhard-Reversat 1996). The litter decomposition of nitrogen-fixing trees positively affect the acceleration of nutrient release and nutrient deposition to promote vegetation development and improve soil properties (Lugo 1992; Ruiz-Jaén & Aide 2005; Lanuza *et al.* 2018), suggesting that *A. mangium* plantation should be used for improving nutrient cycling in mining reclamation. Nutrient cycling is a key process for mining rehabilitation, relating to vegetation succession and soil development in the mining area.

CONCLUSION

A. mangium plantation is an effective way for mining rehabilitation compared to the reference sites. *A. mangium* plantation showed relatively high litter production and litter decomposition rate which could modify the litter quality in the litter mixture. Mixed litter sourced from nitrogen-fixing trees and native trees in plantations can enhance litter decomposition, which can result in higher levels of soil improvement and revegetation. AMS, AMC and MP had high levels of nutrient return compared to that in SF and PF, leading to contributing high levels of soil nutrients to forest floor, particularly N. The litter mixture in MP decomposed more rapidly compared to that in PF with improved the litter quality. A mixed plantation consisting of *A. mangium* and native tree species could be considered for reclamation efforts in mining area and other similar degraded lands.

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REFERENCES

Anderson J, Proctor MJ, Vallack HW. 1983. Ecological studies in four contrasting lowland rain forests in Gunung Mulu National Park, Sarawak: III. Decomposition processes and nutrient losses from leaves litter. J Ecol 71:503-27.

Bell FG, Donnelly L J. 2006. Mining and its impact on the environment. London (UK): Taylor and Francis. 547 p.

Bernhard-Reversat F. 1996. Nitrogen cycling in tree plantations grown on a poor sandy savanna soil in Congo. Appl Soil Ecol 4:161-72.

Bunyavejchewin S. 2001. Ecological studies of tropical semi-evergreen rain forest at Sakaerat, Nakhon Ratchasima, Northeast Thailand, II. Litterfall. In: Silvicultural Research Report. Bangkok (TH): Royal Forest Department.

Berg B, McClaugherty C. 2008. Plant litter decomposition, humus formation, carbon sequestration. Heidelberg (DE): Springer-Verag Berlin.

Celentano D, Zahawi R A, Finegan B, Ostertag R, Cole RJ, Holl KD. 2011. Litterfall dynamics under different tropical forest restoration strategies in Costa Rica. Biotropica 43:278-87.

Chazdon RL. 2014. Second growth: The promise of tropical forest regeneration in an age of deforestation. Chicago (US): University of Chicago Press. 472 p.

Chen L, Xiang W, Wu H, . Lei P, Zhang S, Ouyang S, Deng X, Fang X. 2017. Tree growth traits and social status affect the wood density of pioneer species in secondary subtropical forest. Ecol Evol 7:5366-77.

Cizungu L, Staelens J, Huygens D, Walangululu J, Muhindo D, Cleemput OV, Boeckx P. 2014. Litterfall and leaf litter decomposition in a central African tropical mountain forest and Eucalyptus plantation. For Ecol Manage 326:109-16.

Dutta RK, Agrawal P. 2003. Restoration of opencast coal mine spoil by planting exotic tree species: a case study in dry tropical region. Ecol Eng 21:143-51.

Feng C, Wang Z, Ma Y, Fu S, Chen HYH. 2019. Increased litterfall contributes to carbon and nitrogen accumulation following cessation of anthropogenic disturbances in degraded forests. For Ecol Manage 432:832-9.

Fisher RF, Binkley D. 2000. Ecology and management of forest soils. New York (US): John Wiley and Sons. 456 p.

Gartner TB, Cardon ZG. 2004. Decomposition dynamics in mixed-species leaf litter. Oikos 104:230-46.

Glumphabutr P, Kaitpraneet S, Wachrinrat C. 2007. Aboveground biomass and litterfall of natural evergreen forest in eastern region of Thailand. Kasetsart J (Nat Sci) 41:811-22.

Goma-Tchimbakala J, Bernhard-Reversat F. 2006. Comparison of litter dynamics in three plantations of an indigenous timber-tree species (*Terminalia superba*) and a natural tropical forest in Mayombe, Congo. For Ecol Manage 229:304-13.

Gomes ACS, Luizão FJ. 2012. Leaf and soil nutrients in a chronosequence of secondary-growth forest in Central Amazonia: Implications for restoration of abandoned lands. Res Ecol 20:339-45.

Hättenschwiler S, Coq S, Barantal S, Handa IT. 2011. Leaf traits and decomposition in tropical rainforests: revisiting some commonly held views and towards a new hypothesis. New Phytol 189:950-65.

Inagaki M, Kamo K, Titin J, Jamalung L, Lapongan, Miura S. 2010. Nutrient dynamics through fine litterfall in three plantations in Sabah, Malaysia, in relation to nutrient supply to surface soil. Nutr Cycling Agroecosyst 88:381-95.

Kamo K, Vacharangkura T, Tiyanon S, Viriyabuncha C, Nimpila S, Duangsrisen B, Thaingam R, Sakai M. 2008. Biomass and dry matter production in planted forests and an adjacent secondary forest in the grassland area of Sakaerat, Northeastern Thailand. Tropics 17:209-24.

Koonkhunthod N, Sakurai K, Tanaka S. 2007. Composition and diversity of woody regeneration in a 37-year-old teak (*Tectona grandis* L.) plantation in Northern Thailand. For Ecol Manage 247: 246-54.

Lanuza O, Casanoves F, Zahawi RA, Celentano D, Delgado D, Holl KD. 2018. Litterfall and nutrient dynamics shift in tropical forest restoration sites after a decade of recovery. Biotropica 50(3):491-8.

Lei H, Peng Z, Yigang H, Yang Z. 2015. Vegetation succession and soil infiltration characteristics under different aged refuse dumps at the Heidaigou open coal mine. Glob Ecol Conserv 4:255-63.

Liao JH, Wang HH, Tsai CC, Hseu ZY. 2006. Litter production, decomposition and nutrient return of uplifted coral reef tropical forest. For Ecol Manage 235:174-85.

Liu C, Liu Y, Guo K, Zhao H, Qiao X, Wang S, Zhang L, Cai X. 2016. Mixing litter from deciduous and evergreen trees enhances decomposition in a subtropical karst forest in southwestern China. Soil Biol Biochem 101:44-54.

Lugo AE. 1992. Comparison of tropical tree plantations with secondary forest of similar age. Ecol Monogr 62:1-41.

Martpalakorn M. 1990. Tree species trials on mined spoils at Amphoe Takuapa, Changwat Phangnga [Dissertation]. Bangkok (TH): Kasetsart University Retrieved from Kasetsart University.

Moura PM, Althoff TD, Oliveira RA, Souto JS, Souto PC, Menezes RSC, Sampaio EVSB. 2016. Carbon and nutrient fluxes through litterfall at four succession stages of Caatinga dry forest in Northeastern Brazil. Nutr Cycling in Agroecosyst 105:25-38.

Ngugi MR, Fechner N, Neldner VJ, Dwnnis PG. 2020. Successional dynamics of soil fungal diversity along a restoration chronosequence post-coal mining. Res Ecol 28:543-52.

Oktavia D, Setiadi Y, Hilwan I. 2015. The comparison of soil properties in heath forest and post-tin mined land: basic for ecosystem restoration. Procedia Environ Sci 28:124-31.

Olson JS. 1963. Energy storage and the balance of producers and decomposers in ecological systems. Ecol 44:322-31.

Parrotta JA. 1999. Productivity, nutrient cycling, and succession in single- and mixed-species plantations of *Casuarina equisetifolia*, *Eucalyptus robusta*, and *Leucaena leucocephala* in Puerto Rico. For Ecol Manage 124:45-77.

Parsons SA, Congdon RA. 2008. Plant litter decomposition and nutrient cycling in North Queensland tropical rain-forest communities of differing successional status. J Trop Ecol 24: 317-27.

Paudel E, Dossa GGO, Xu J, Harrison RD. 2015. Litterfall and nutrient return along a disturbance gradient in a tropical montane forest. For Ecol Manage 353:97-106.

Rai A, Singh AK, Ghosal N, Singh N. 2016. Understanding the effectiveness of litter from tropical dry forests for the restoration of degraded lands. Ecol Eng 93:76-81.

Reichl C, Schatz M, Zsak G. 2017. World mining data. Federal Ministry of Science, Research and Economy, Vienna (Austria). 255 p.

Ruiz-Jaén MC, Aide TM. 2005. Vegetation structure, species diversity, and ecosystem processes as measures of restoration success. For Ecol Manage 218:159-73.

Scherer-Lorenzen M, Bonilla JL, Potvin C. 2007. Tree species richness affects litter production and decomposition rates in a tropical biodiversity experiment. Oikos 116:2108-24.

Singh AN, Raghubanshi AS, Singh JS. 2004. Impact of native tree plantations on mine spoil in a dry tropical environment. For Ecol and Manage 187:49-60.

Tang JW, Cao M, Zhang JH, Li MH. 2010. Litterfall production, decomposition and nutrient use efficiency varies with tropical forest types in Xishuangbanna, SW China: a 10 year study. Plant Soil 335:271-88.

Thaiutsa B, Rungruansilp C. 1990. Impacts of tin mining on soil properties. Thai J For 9:73-82.

Triadiati S, Tjitrosemito E, Guhardja E, Sudarsono I, Qayim I, Leuschner C. 2011. Litterfall production and leaf litter decomposition at natural forest and cacao agroforestry in central Sulawesi, Indonesia. Asian J Biol Sci 4:221-34.

Tripathi N, Singh RS, Hills CD. 2016. Reclamation of mine-impacted land for ecosystem recovery. London (UK): John Wiley and Sons.

Trogisch S, He JS, Hector A, Scherer-Lorenzen M. 2016. Impact of species diversity, stand age and environmental factors on leaf litter decomposition in subtropical forests in China. Plant Soil 400: 337-50.

Wongprom J, Poolsiri R, Diloksumpun S, Ngernsaengsaruay C. 2020. Soil properties and tree composition in a 27-year old *Acacia mangium* Willd. plantation on abandoned mining area at Phangnga Forestry Research Station. Biotropia 27:125-33.

Xuluc-Tolosa FJ, Vester HFM, Ramı´rez-Marcial N, Castellanos-Albores J, Lawrence D. 2003. Leaf litter decomposition of tree species in three successional phases of tropical dry secondary forest in Campeche, Mexico. For Ecol Manage 174:401-12.

Zhang Y, Yang JY, Wu HL, Shi CQ, Zhang CL, D. Li X, Feng MM. 2014. Dynamic changes in soil and vegetation during varying ecological recovery conditions of abandoned mines in Beijing. Ecol Eng 73:676-83.

Zhao Z, Shahrour I, Bai Z, Fan W, Feng L, Li H. 2013. Soil development in opencast coal mine spoils reclaimed for 1-13 years in the West-Northern Loess Plateau of China. Eur J Soil Biol 55:40-6.

Zhong Y, Yan W, Wang R, Shangguan Z. 2017. Differential responses of litter decomposition to nutrient addition and soil water availability with long-term vegetation recovery. Biol Fertil Soils 53:939-49.

Zhou G, Guan L, Wei X, Zhang D, Zhang Q, Yan J, Wen D, Liu J, Liu S, Huang Z, Kong G, Mo J, Yu Q. 2006. Litterfall production along successional and altitudinal gradients of subtropical monsoon evergreen forest broadleaved forests in Guangdong, China. Plant Ecol 188:77-89.

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NEUSTON DIVERSITY AND DENSITY AS BIOINDICATOR FOR WATER QUALITY

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ABSTRACT

Lakes and waterfalls are freshwater ecosystems having important roles in ecology, tourism and economic aspects. Among living organisms existing in lakes and waterfalls is neuston. Neuston lives on the surface and below the surface of the waters. Neuston can be used as a bioindicator for water quality due to the neuston's high level of sensitivity toward pollutants. The purpose of this study was to determine the density and diversity of neuston as a bioindicator for water quality in the lake and waterfall. The methods used were survey and observation. The study was carried out by using purposive sampling at two locations, namely the lake and waterfall of Situ Gunung, Sukabumi, with a sampling area of 1 x 1 m^2. The samples obtained were put into bottles containing 70% alcohol to be identified in the laboratory. Environmental parameters measured were air and water temperature, water pH, water depth, turbidity, water flow velocity, dissolved oxygen, substrate and weather conditions. The results obtained indicated that the lake and waterfall of Situ Gunung, Sukabumi had highest neuston densities were shown by *Gerris lacustris* and *Dineutus assimilis*. The lowest neuston densities were shown by *Metrobates hesperius*, *Gerris comatus*, *Aquarius remiges* and *Trepobates pictus*. This study showed that the environmental parameters of the lake and waterfall of Situ Gunung, Sukabumi can still support the survival of the existing neustons.

Keyword: density, diversity, neuston

INTRODUCTION

Freshwater ecosystems are divided into lentic/flooded freshwater ecosystems and lotic/flowing freshwater ecosystems. The examples of lentic freshwater ecosystems are lakes and swamps, while that of lotic freshwater ecosystems are waterfalls and rivers (Diantari *et al.* 2017; Ramadhan *et al.* 2016). The existence of the lake is considered important because it has an ecological, social, and economic roles for the surrounding environment (Postel & Carpenter 1997; Chen *et al.* 2020; Heino *et al.* 2021; Robert *et al.* 2020). The lake also acts as a habitat for several living organisms, such as neuston (Asnil *et al.* 2013; Ramadhan *et al.* 2016).

According to Mulyono (2018), neuston floats on water (epineuston) or below the water surface (hyponeuston). Hyponeuston lives at a depth of about 0 - 10 cm (Rumnasih 2016). Neuston has a level of sensitivity to several contaminants so neuston can be used as a direct indicator for water contamination (Oktarina 2015) and as a bioindicator for water quality. The upper part of the waters is the most susceptible part to environmental exposure, which in turn will impact the neuston. Pollution often causes the formation of artificial stratification in lakes, where a body of water (hypolimnetic zone) is isolated. However, the hypolimnetic zone caused the contaminants trapped within the bottom of the waters and sediment masses (Sutrisno & Hamdani 2013; Williams 2001; Winton *et al.* 2019; Perron *et al.* 2014; Donyinah 2019).

Waterfalls are a form of lotic freshwater ecosystem and are generally used as natural tourist objects (Siswantini & Mulyana 2017). The waterfall and lake in the Situ Gunung area, Sukabumi have an important role as one of

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tourist attractions in Kadudampit District, Sukabumi, West Java Province, Indonesia (Soetopo 2011). Situ Gunung as a tourist object cannot be separated from tourists activities which will affect the surrounding environment, including the neuston community. Therefore, the purpose of this study was to determine environmental factors influencing the density and diversity of neuston as water quality bioindicator in the lake and waterfall in Situ Gunung, Sukabumi.

MATERIALS AND METHODS

The study was conducted in June 2021 at Situ Gunung, Gunung Gede Pangrango, Kadudampit District, Sukabumi. The methods used were survey and observation. The tools used were rope, wooden stick, measuring tape, thermometer, turbidimeter, DO meter, loop, field guide as an identification guide, stopwatch, plastic bottle, sample bottle, sample plastic, camera, net 1 x 1 m^2, identification key book and stationery. The materials used were 70% alcohol, labeling paper and universal pH indicator paper (1 - 14).

The study was conducted in two study locations, namely lake and waterfall in the shallowest part. Each location was represented by three observation stations based on differences in environmental conditions. Sampling was carried out three times at each station. Neuston collection was carried out at locations and stations that were purposively determined as a pick-up point sized 1 x 1 m^2. Neuston samples were taken by using a 1 x 1 m^2 net which was placed on the water surface. The net captured neustons which went through the filtration following the horizontal movement of the surface water in the lake and waterfall. Subsequently, the neustons sample was put into a bottle containing 70% alcohol. Samples were identified in the field and photographed for documentation.

Environmental parameters measured were air and water temperature, water pH, water depth, water turbidity, water flow velocity, dissolved oxygen, substrate and weather conditions. The air temperature was measured using a calibrated air thermometer. Water temperature measurements were carried out using a DO meter. The pH of the water was measured using universal pH indicator paper (1 - 14). The water depth of the lake and waterfall was measured using a long wooden stick. The mark of the water depth was then measured by using a measuring tape. Water turbidity was measured by using a calibrated turbidity meter. Water flow velocity was measured by using floating method from the edge of the lake and waterfall. A rope was tied to a bottle and a 1-meter-long wooden stick. The bottle was floating at the water surface. The time taken from the moment the bottle was tied up to the wooden stick until the rope was stretched straight, was recorded by a stopwatch. The water flow velocity is expressed in m/s unit. The formula for calculating the water flow velocity is as follows:

$$\text{Flow Velocity} = \frac{\text{Rope Length (m)}}{\text{Time (s)}}$$

Dissolved oxygen was measured three times using a DO meter. The substrate is the surface on which an organism lives. The substrate sample of the lake was taken using the Ekman Grab, while the substrate of the waterfall can be seen directly because the water depth is quite shallow. The substrate was determined by looking at its composition. The types of water substrates are sand, mud, rocks, lime, and others. Weather was determined by looking at the weather situation at the lake and waterfall environment. There are three kinds of weather conditions, namely sunny, cloudy, and rainy. Data analysis was carried out by determining the Simpson index using the formula as follows:

1. Density

$$B = \frac{T \times P}{A \times S}$$

where:
T = quadrant area (1 m^2 = 10,000 cm^2)
P = area of the taking transect (m^2)
A = number of individual species
S = number of taking transects

2. Diversity

$$D_s = 1 - D \rightarrow D_s = 1 - \frac{\Sigma\, Ni\,(ni - 1)}{N\,(N - 1)}$$

where:
Ds = Simpson's diversity index

D = dominant index
ni = number of individuals species
N = total number of individuals

RESULTS AND DISCUSSION

There were six species of neuston found in the lake and waterfall of Situ Gunung. Four species were existed in the lake, namely *Gerris lacustris*, *Metrobates hesperius*, *Gerris comatus*, and *Aquarius remigis* (Fig. 1), while two species were found in the waterfall, namely *Dineutus assimilis* and *Trepobates pictus* (Fig. 2).

Neuston species found in the lake and waterfall are different, depending on the habitat. *Gerris lacustris* and *Gerris comatus* are found in lakes due to their lentic habitat preferences. According to Ye *et al.* (2017), *Gerris* species inhabit several lentic habitats such as ponds, lakes and backwaters of streams. The habitat preference of *Gerris lacustris* is a high level of water depth, covered with vegetation, which is in agreement with a study conducted by Olosutean and Ilie (2013) who found that *Gerris lacustris* is correlated with water depth and have adaptation benefits from the presence of vegetation cover. The surface of the lakeside of Situ Gunung is covered with aquatic plants and the depth is relatively higher (58 - 93 cm) compared to the water depth of the waterfall (25 - 64 cm). In addition, *Gerris comatus* is a characteristic organism for lentic water. *Gerris comatus* is often found together with *Gerris marginatus* and *Gerris buenoi* (Damgaard *et al.* 2014; Pintar & William 2020). However, the last two species mentioned are not found in the lake.

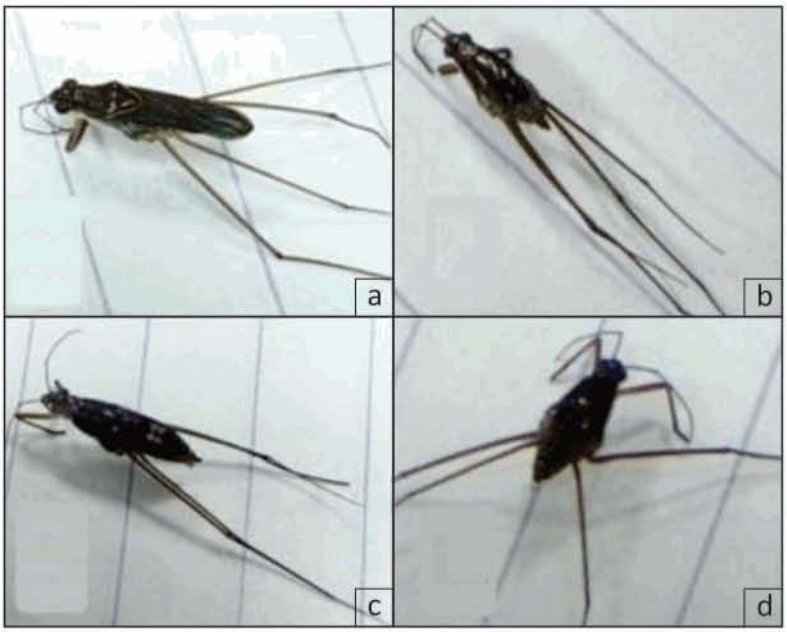

Figure 1 Neuston species found in the lake of Situ Gunung
Notes: a. *Gerris lacustris*; b. *Metrobates hesperius*; c. *Gerris comatus*; d. *Aquarius remigis*

Figure 2 Neuston species found in the waterfall of Situ Gunung
Notes: a. *Trepobates pictus*; b) *Dineutus assimilis*

Aquarius remigis were found in the lake of Situ Gunung, which fact is different from the statement of Ye *et al.* (2017) that *Aquarius* species are mostly confined to lotic habitats, such as water springs and rivers. *Aquarius* species can live in two different kinds of freshwater, both lentic and lotic. *Aquarius* species are semi-aquatic insects and opportunistic predators that live on the water surface of lakes, rivers, and the border between rivers and lentic habitats (Ditrich & Papáček 2016; Guterres *et al.* 2019). *Metrobates hesperius* were found in the lake because there are aquatic plants on the surface of the lakeside of Situ Gunung. *Metrobates hesperius* lays eggs on the leaves of the floating aquatic plants (Taylor 2009; Ikawa *et al.* 2012; Finet *et al.* 2018). The lake as a habitat supports the survival and distribution of these species.

Dineutus assimilis and *Trepobates pictus* were found on the surface of a waterfall. *Dineutus assimilis* prefers both lotic and lentic waters, such as river surfaces, lakeshores and lakes (Gustafson & Miller 2015; MacLean 2013; Webster & DeMerchant 2012). Habitat preferences of *Trepobates pictus* were waters with slow water flow with eutrophic, muddy and rocky conditions in lotic waters (Naranjo *et al.* 2010; Taylor & McPherson 2006; Wooden 2019; Chordas 2017). The diversity index value (Ds) of neuston species obtained in the lake and waterfall for each station in Situ Gunung is presented in Table 1. Diversity index of 0.50 means low diversity, diversity index value of 0.50 to 0.75 means moderate diversity index, while diversity index of 0.75 to 1 means high diversity index (Nento *et al.* 2018).

Table 1 Neuston diversity and total individuals in the lake and waterfall of Situ Gunung

Location	Station 1		Station 2		Station 3	
	Ds	TI	Ds	TI	Ds	TI
Lake	0.00	4	0.11	18	0.70	5
Waterfall	0.47	10	0.00	6	0.48	7

Notes: Ds = Diversity, TI = Total Individuals

Table 1 shows that neuston diversity index value (Ds) varies among stations. The highest neuston diversity index in the lake occurred at station 3 with a value of 0.70 (moderate) with a total of 5 inviduals and 3 neuston species, namely *Gerris lacustris*, *Gerris comatus* and *Aquarius remigis*. Neuston diversity index value in the lake at station 2 was 0.11 (low) with a total of 18 individuals and 2 neuston species, namely *Gerris lacustris* and *Metrobates hesperius*. The lowest neuston diversity index in the lake occurred at station 1 with a value of 0.00 (low). In station 1, only one neuston species was found, namely *Gerris lacustris* with a total of 4 individuals. The highest neuston diversity index in the waterfall occurred at station 3 with a value of 0.48 (low), where 2 neuston species were found, namely *Dineutus assimilis* and *Trepobates pictus* with a total of 7 individuals. The lowest neuston diversity index in the waterfall occurred at station 2 with a value of 0.00 (low), where 1 neuston species was found, namely *Dineutus assimilis* with a total of 6 individuals. The neuston diversity index in the waterfall at station 1 was 0.47 (low) with a total of 10 individuals and 2 neuston species, namely *Dineutus assimilis* and *Trepobates pictus*. The different values of diversity index with total individuals obtained was due to dominant species in the lake and waterfall of Situ Gunung, namely *Gerris lacustris* and *Dineutus assimilis*.

Table 2 Neuston density in the lake and waterfall of Situ Gunung

Location	Neutston species	Neuston density (individu/m^2)
Lake	*Gerris lacustris*	0.24
	Metrobates hesperius	0.01
	Gerris comatus	0.01
	Aquarius remigis	0.01
Waterfall	*Dineutus assimilis*	0.18
	Trepobates pictus	0.05

The highest neuston density neuston in the lake was shown by *Gerris lacustris* with a value of 0.24 individu/m^2. The three other neuston species, namely *Metrobates hesperius*, *Gerris comatus*, and *Aquarius remigis* showed the same density value of 0.01 individu/m^2 (Table 2). On the lake surface of Situ Gunung at stations 1 to 3, there are aquatic plants. *Gerris lacustris* prefers to live on aquatic plants available on the lake surface of Situ Gunung. According to Yee (2016), *Gerris lacustris* spends most of its life on the surface of the water. In the lake of Situ Gunung there were two species of *Gerris*, namely *Gerris lacustris* and *Gerris comatus*. *Gerris* species can be found in almost all aquatic habitats from water springs to tropical seas (Yurtseven *et al.* 2016). At the waterfall of Situ Gunung, the

highest neuston density was shown by *Dineutus assimilis* with a value of 0.18 individu/m^2 and *Trepobates pictus* with a value of 0.05 individu/m^2. The common aquatic insects found in Indonesian freshwater are of the Order Odonata, Coleoptera, Trichoptera, Hemiptera, Ephemeroptera, Plecoptera and Lepidoptera (Mahajoeno *et al.* 2001; Candra *et al.* 2014). In the lake and waterfall of Situ Gunung, we found Orders Hemiptera and Coleoptera.

Every species found in the lake of Situ Gunung belongs to Gerridae family. According to Dwitawati *et al.* (2015), Gerridae family is classified as having a low tolerance for pollutants. Meanwhile, *Gerris lacustris*, *Gerris comatus*, *Metrobates hesperius* and *Aquarius remigis* belong to the Order Hemiptera. Hemiptera insects often move rather than being settled in an unwanted location (Mercer *et al.* 2017; Peterson *et al.* 2017; Wooden 2019). The predominant presence of Hemiptera insects indicates that the lake is relatively less polluted (Majumder 2013). The pollution that contaminates the lake of Situ Gunung is thought to come from household wastes and human recreational activities. Aquatic insects are a good bioindicator for detecting pollution. Aquatic insects are also useful for fish food and biocontrol agents (Dalal & Gupta 2016; Ito *et al.* 2017). Therefore, long-term monitoring of aquatic insects is needed to evaluate water quality.

Table 3 shows that the pH values in the lake of Situ Gunung for all stations are relatively constant, namely 6 (acid). The standard pH for clean water quality ranges from 6.5 to 9.0. The pH value in the lake of Situ Gunung which is below the clean water standard is suspected to have been polluted by household wastes due to the closeness with the mainland, hence acidic pH value. The acidic pH value is not within the suitable environment for the neuston's life. Neuston can develop well in the pH range of 6.8-8.5 (Gundo 2010; Pratami *et al.* 2018). pH affects dissolved oxygen levels. The lowest dissolved oxygen level for the lake of Situ Gunung location was found at station 2, which was 3.28 mg/L. The acidic pH causes an increase in toxic substances in the water and decreases dissolved oxygen levels (Pratami *et al.* 2018).

Dissolved oxygen plays an important role in the respiration process of most aquatic organisms (Hariyani *et al.* 2017; Pratami *et al.* 2018). The average of dissolved oxygen level in the lake of Situ Gunung was 3.62 mg/L. Dissolved oxygen levels also indicate the level of pollution waters. Dissolved oxygen levels below 5 ppm mean low pollution levels (Hariyani *et al.* 2017). The low dissolved oxygen level shown in the lake of Situ Gunung indicated that the pollution level was low enabling neuston organisms such as water insects to obtain the needed oxygen for the respiration process.

The lake of Situ Gunung showed the highest water turbidity value at station 1, which was 11.97 NTU. The high value of water turbidity is presumably caused by the existing fishing activities by humans and aquatic plants on the lake surface at station 1. Penetration of sunlight into the lake waters can be hindered by the high value of water turbidity (Pratami *et al.* 2018). The lowest value of water turbidity was shown at station 2, which was 4.44 NTU. The different value of water turbidity at station 2 compared to the other two stations is presumably caused by the utilization of station 2, which function is docking place for boats. Also, only a few of aquatic plants were existing in station 2.

The measurement of environmental parameters of the waterfall of Situ Gunung showed air and water temperatures of 20.7 °C and 17.9 °C, respectively, indicating a cold-water habitat for insects (Table 4). In the environment of the lake of Situ Gunung, the air and water temperatures of 29 °C and 28 °C, respectively, indicating a warm-habitat for insects (Table 3).

According to Mujiono *et al.* (2019), the higher the place is, the colder the temperature. The average of water pH of the waterfall of Situ Gunung is 6 (acidic) with average of Dissolved Oxygen value of 7.77 mg/L. Dissolved oxygen levels are high in the waterfall because the waterfall is located on a hill surrounded by natural forests and has low pollution levels. Meanwhile, the lake water is murky indicating water pollution that affects dissolved oxygen levels. In addition, the murky water can cause the increase of oxygen uptake from the air into the water causing the increase of dissolved oxygen in the water (Diantari *et al.* 2017; Mujiono *et al.* 2019; Irby *et al.* 2015).

Table 3 Environmental parameters obtained from the lake of Situ Gunung

Environmental parameter	Station			Mean
	1	2	3	
Water pH	6	6	6	6
Air temperature (°C)	29	29	29	29
Water temperature (°C)	28.03	27.93	27.93	27.97
Water depth (cm)	58	71	93	74
Water turbidity (NTU)	11.97	4.44	11.85	9.42
Water flow velocity (m/s)	0.009	0.022	0.0085	0.013
Dissolved Oxygen (mg/L)	4.24	3.28	3.33	3.62
Substrate	Mud and rocky	Mud and rocky	Mud and rocky	
Weather	Cloudy and rainy	Cloudy and rainy	Cloudy and rainy	

Table 4 Environmental parameters obtained from the waterfall of Situ Gunung

Environment	Station			Mean
	1	2	3	
Water pH	6	6	6	6
Air temperature (°C)	20.8	20.8	20.5	20.7
Water temperature (°C)	17.9	17.9	17.9	17.9
Water depth (cm)	25	53.33	64.33	47.56
Water turbidity (NTU)	0.41	0.46	0.51	0.46
Water flow velocity (m/s)	0.49	0.58	0.13	0.40
Dissolved Oxygen (mg/L)	7.54	7.71	8.05	7.77
Substrate	Rocky	Rocky	Rocky	
Weather	Sunny	Sunny	Sunny	

The water flow of the stations at waterfall of Situ Gunung showed an average of water flow velocity of 0.40 m/s which affects the presence of swimming-type aquatic insects. Due to the high value of water flow velocity and the presence of rocky substrate, the water insects hide behind rocks (Leba *et al.* 2013). According to Diantari *et al.* (2017), the rocky substrate can affect the water flow velocity in a stream. The lake water's flow velocity of 0.01 m/s causes the lake to be predominated by neustons with floating ability. The average water turbidity of the waterfall of Situ Gunung was 0.46 NTU with an average water depth of 47.56 cm. Meanwhile, the lake showed an average turbidity of 9.42 NTU with an average water depth of 74 cm (Table 3). Our study showed that sunlight easily penetrated the waterfall. However, the murky lake water hindered the sunlight penetration into the lake. Small value of water turbidity can support filter-feeder organisms and affect the activities of the existing Orders Hemiptera and Coleoptera (Ebenebe *et al.* 2016; Marpaung *et al.* 2014).

CONCLUSION

Six neuston species were identified in Situ Gunung, namely four species in the lake of Situ Gunung and two species in the waterfalls of Situ Gunung. The four neuston species in the lake namely *Gerris lacustris*, *Metrobates hesperius*, *Gerris comatus* and *Aquarius remigis,* while the two neuston species waterfalls namely *Dineutus assimilis* and *Trepobates pictus*. The highest neuston densities in the lake and waterfalls were represented by *Gerris lacustris* and *Dineutus assimilis*, respectively. Neuston diversity in the lake and waterfalls of Situ Gunung was low. Environmental parameters of the lake and waterfalls of Situ Gunung were still in the normal range, except low water pH indicating polluted waters environment. Therefore, long-term monitoring at the waters of Situ Gunung is needed for evaluating water quality parameters that support the life of aquatic organisms.

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REFERENCES

Asnil A, Mudikdjo K, Hardjoamidjojo S, Ismail A. 2013. Analisis kebijakan pemanfaatan sumberdaya danau yang berkelanjutan (Studi kasus Danau Maninjau Sumatera Barat). J Nat Res Environ Manage 3(1):1-9.

Candra Y, Langoy M, Koneri R, Singkoh MF. 2014. Kelimpahan serangga air di Sungai Toraut Sulawesi Utara. Jurnal MIPA 3(2):74-8.

Chen Y, Ke X, Min M, Cheng P. 2020. Disparity in perceptions of social values for ecosystem services of urban green space: A case study in the East Lake Scenic Area, Wuhan. Public Health Front 8:1-11.

Chordas SW. 2017. Literature record checklist of true bugs (Hemiptera) for Arkansas, USA. Journal of the Arkansas Academy of Science 71:224-31.

Dalal A, Gupta S. 2016. A comparative study of the aquatic insect diversity of two ponds located in Cachar District, Assam, India. Turk J Zool 40(3):392-401.

Damgaard J, Moreira FFF, Zettel H, Weir TA. 2014. Molecular phylogeny of the pond skaters (Gerrinae). Discussion of the fossil record and a checklist of species assigned to the subfamily (Hemiptera: Heteroptera: Gerridae). Insect Syst Evol 45(3):251-81.

Diantari NPR, Ahyadi H, Rohyani IS, Suana IW. 2017. Diversity of Ephemeroptera, Plecoptera, and Trichoptera as bioindicator of water quality in Jangkok River, West Nusa Tenggara. Indonesian Journal of Entomology 14(3):135-42.

Ditrich T, Papáček M. 2016. Differences in prey capture in semiaquatic bugs (Heteroptera: Gerromorpha). Entomol Sci 19(1):34-41.

Donyinah SK. 2019. Persistent Organic Pollutants. London (UK): IntechOpen Ltd. 108 p.

Dwitawati DA, Sulistyarsi A, Widiyanto J. 2015. Biomonitoring kualitas air Sungai Gandong dengan bioindikator makroinvertebrata sebagai bahan petunjuk praktikum pada pokok bahasan pencemaran lingkungan SMP Kelas VII. Florea: Jurnal Biologi dan Pembelajarannya 2(1):41-6.

Ebenebe CI, Ihuoma JN, Ononye BU, Ufele AN. 2016. Effect of physicochemical properties of water on aquatic insect communities of a stream in Nnamdi Azikiwe University, Awka, Nigeria. Int J Entomol Res 1(7):32-6.

Finet C, Decaras A, Armise´n D, Khila A. 2018. The Achaete-Scute complex contains a single gene that controls bristle development in the semi-aquatic bugs. *Proc. R. Soc. B.* 285: 20182387.

Gundo MT. 2010. Kerapatan, keanekaragaman dan pola penyebaran gastropoda air tawar di Perairan Danau Poso. Media Litbang Sulteng 3(2):137-43.

Guterres APM, Cunha EJ, Godoy BS, Silva RR, Juen L. 2019. Co¯occurrence patterns and morphological similarity of semiaquatic insects (Hemiptera: Gerromorpha) in Streams of Eastern Amazonia. Ecol Entomol 45(1):1-12.

Gustafson G, Miller K. 2015. The new world whirligig beetles of the genus *Dineutus* Macleay, 1825 (Coleoptera, Gyrinidae, Gyrininae, Dineutini). ZooKeys 476:1-135.

Hariyani D, Slamet A, Santri DJ. 2017. Jenis-jenis protista di Danau Teluk Gelam Kabupaten OKI Provinsi Sumatera Selatan. Jurnal Pembelajaran Biologi: Kajian Biologi dan Pembelajarannya 4(2):126-36.

Heino J, Alahuhta J, Bini LM, Cai Y, Heiskanen AS, Hellsten S, Kortelainen P, Kotamäki N, Tolonen KT, Vihervaara P, Vilmi A, Angeler DG. 2021. Lakes in the era of global change: moving beyond single-lake thinking in maintaining biodiversity and ecosystem services. Biol Rev 96:89-106.

Ikawa T, Okabe H, Cheng L. 2012. Skaters of the seas–comparative ecology of nearshore and pelagic Halobates species (Hemiptera: Gerridae), with special reference to Japanese species. Mar Biol Res 8(10):915-36.

Irby SD, Marjorie AM, Friedrichs DF, Hinson KE. 2018. The competing impacts of climate change and nutrient reductions on dissolved oxygen in Chesapeake Bay. Biogeosciences 15: 2649-68.

Ito EE, Ugbomeh IL. 2017. Environmental effect of resort centres on the distribution of aquatic insect fauna in Ethiope River, Delta State, Nigeria. J Coast Life Med 5(11):468-73.

Leba GV, Koneri R, Papu A. 2013. Keanekaragaman serangga air di Sungai Pajowa Kabupaten Minahasa, Sulawesi Utara. Jurnal MIPA 2(2):73-8.

MacLean DB. 2013. Inventory of Aquatic and Semiaquatic Coleoptera from the Grand Portage Indian Reservation, Cook County, Minnesota Portage Indian Reservation, Cook County, Minnesota. Gt Lakes Entomol 46(1):104-15.

Mahajoeno E, Efendi M, Ardiansyah. 2001. Keanekaragaman larva insekta pada sungai-sungai kecil di hutan Jobolarangan. Biodiversitas 2:133-9.

Majumder J, Das RK, Majumder P, Ghosh D, Agarwala BK. 2013. Aquatic insect fauna and diversity in urban fresh water lakes of Tripura, Northeast India. Middle-East J Sci Res 13(1):25-32.

Marpaung SM, Muhammad F, Hidayat JW. 2014. Keanekaragaman dan kemelimpahan larva insekta akuatik sebagai bioindikator kualitas air di Sungai Garang, Semarang. Jurnal Akademika Biologi 3(4):1-8.

Mercer N, Kaller MD, Stout MJ. 2017. Diversity of arthropods in farmed wetlands in the Gulf of Mexico Coastal Plain and effects of detrital subsidies. J Fresh Ecol 32(1):163-78.

Mujiono N, Afriansyah, A, Putera, AK, Atmowidi, T, Priawandiputra, W. 2019. Keanekaragaman dan komposisi keong air tawar (mollusca: gastropoda) di beberapa situ Kabupaten Bogor dan Kabupaten Sukabumi. Limnotek: Perairan Darat Tropis di Indonesia. 26 (2): 65-76.

Mulyono M, Firdaus R, Alka CM. 2018. Sumber daya hayati laut Indonesia: Sebuah pengantar sumber daya hayati laut Indonesia Volume 1. Jakarta (ID): STP Press.

Naranjo C, Riviaux SM, Moreira FFF, Court RC. 2010. Taxonomy and distribution of aquatic and semiaquatic Heteroptera (Insecta) from Cuba. Rev. Biol. Trop. 58(3):897-907.

Nento R, Sahami F, Nursinar S. 2013. Kelimpahan, keanekaragaman dan kemerataan Gastropoda di ekosistem mangrove Pulau Dudepo, Kecamatan Anggrek, Kabupaten Gorontalo Utara. The NIKe Journal 1(1):41-7.

Oktarina A, Syamsudin TS. 2015. Keanekaragaman dan distribusi makrozoobentos di perairan lotik dan lentik Kawasan Kampus Institut Teknologi Bandung, Jatinangor Sumedang, Jawa Barat. Prosiding Seminar Nasional Masyarakat Biodiversitas Indonesia. 12 September 2015, Jakarta, Indonesia: p. 227-35.

Olosutean H, Ilie DM. 2013. *Gerris lacustris* (Linaeus 1758) and *Gerris costae* (Herrich-Schäffer 1850) species-habitat relations on montainous tributaries of Viseu River (Maramures, Romania). Transylv Rev Syst Ecol Res 15(1):11-18.

Perron T, Chételat J, Gunn J, Beisner BE, Amyot M. 2014. Effects of experimental thermocline and oxycline deepening on methylmercury bioaccumulation in a Canadian Shield Lake. Environ Sci Technol 48(5):2626-34.

Peterson MG, Lunde KB, Chiu MC, Resh VH. 2017. Seasonal progression of aquatic organisms in a temporary wetland in northern California. West N Am Nat 77(2):176-88.

Pintar MR, William JRJr. 2020. A comparison of aquatic and semiaquatic Heteroptera (Hemiptera) inhabiting natural habitats and experimental mesocosms at the University of Mississippi field station. Aquat Insects 41(1):76-84.

Postel S, Carpenter S. 1997. Freshwater Ecosystem Services. In Daily, G. (Ed). Nature's Services: Washington DC (US): Island Press. p. 195-214.

Pratami VAY, Setyono P, Sunarto S. 2018. Keanekaragaman, zonasi serta overlay persebaran bentos di Sungai Keyang, Ponorogo, Jawa Timur. DEPIK Jurnal Ilmu-Ilmu Perairan, Pesisir dan Perikanan 7(2):127-38.

Ramadhan F, Rijaluddin AF, Assuyuti M. 2016. Studi indeks saprobik dan komposisi fitoplankton pada musim hujan di Situ Gunung, Sukabumi, Jawa Barat. Al-Kauniyah 9(2):95-102.

Robert WS, Bonnie K, Stephen P, Rajendra P, Kirsten R, Maggie R. 2020. Ecosystem services of earth's largest freshwater lakes. Ecosyst Serv 41:101046.

Rumnasih A. 2016. Perbandingan komunitas zooplankton di daerah litoral dengan Estuari Cipatireman, Pantai Sindangkerta, Kecamatan Cipatujah, Kabupaten Tasikmalaya. [Skripsi]. Bandung (ID): Fakultas Keguruan dan Ilmu Pendidikan, Universitas Pasundan.

Siswantini W, Ayuni D, Mulyana A. 2017. Pengaruh komunikasi pemasaran, pengalaman dan kualitas jasa terhadap citra dan kepuasan serta dampaknya pada loyalitas wisatawan nusantara (survei tempat rekreasi air terjun di Kabupaten Bogor). Jurnal Organisasi dan Manajemen 13(1):71-85.

Soeprobowati TR, Hidayat JW, Baskoro K. 2011. Diatom epipelik sebagai bioindikator kualitas perairan Danau Rawa Pening. Jurnal Sains dan Matematika 19(4):107-18.

Soetopo A. 2011. Mengenal Lebih Dekat: Wisata Alam Indonesia. Jakarta (ID): Pacu Minat Baca.

Sutrisno S, Hamdani A. 2013. Pengamatan pola stratifikasi di Danau Maninjau sebagai potensi tubo belerang. Limnotek: perairan darat tropis di Indonesia 20(2):129-40.

Taylor SJ, McPherson JE. 2006. Gerromorpha (Hemiptera: Heteroptera) in Southern Illinois: Species assemblages and habitats. Gt Lakes Entomol 39(1):1-26.

Taylor SJ. 2009. Concurrent phenologies of three semiaquatic bugs (Heteroptera: Gerridae, Veliidae) on a small river in Central Illinois, USA. Psyche 2009:1-5. doi:10.1155/2009/562471

Webster RP, DeMerchant I. 2012. New Coleoptera records from New Brunswick, Canada: Gyrinidae, Carabidae, and Dytiscidae. ZooKeys 179:1-10.

Williams W. 2001. Anthropogenic salinisation of inland waters. Hydrobiologia 466:329-37.

Winton RS, Calamita E, Wehrli B. 2019. Reviews and syntheses: Dams, water quality and tropical reservoir stratification. Biogeosciences 16:1657-71.

Wirosarjono S. 1974. Masalah-masalah yang dihadapi dalam penyusunan kriteria kualitas air guna berbagai peruntukan. PPMKL-DKI Jaya, Seminar Pengelolaan Sumber Daya Air. Bandung (ID): Lembaga Ekologi Universitas Padjadjaran. p. 27-9.

Wooden PLS. 2019. Influence of the annual flood pulse of the Atchafalaya River and updates on the ecology of floodplain aquatic and semi-aquatic hemiptera and their associates. [Thesis]. Baton Rouge (US): Louisiana State University.

Yee KL. 2016. Occurrence of aquatic Macroinvertebrates from Paleik"IN", Sintkaing Township. Yadanabon University Research Journal 7(1):1-12.

Ye Z, Zhen Y, Damgaard J, Chen P, Zhu L, Zheng C, Bu W. 2018. Biogeography and diversification of Holarctic water striders: Cenozoic temperature variation, habitat shifting and multiple intercontinental dispersals. Syst Entomol 43(1): 19-30.

Yurtseven I, Serengil Y, Pamukçu P. 2016. Seasonal changes in stream water quality and its effects on macroinvertebrate assemblages in a Forested watershed. Appl Ecol Environ Res 14(1):175-88.

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a. Sourgrass (*Paspalum conjugatum*)
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Zhang J. 2016a. Coinfection with type 1 and type 2 PRRSV. Vet Rec 178(12):288-90.

Zhang J. 2016b. Porcine delta corona virus: Overview of infection dynamics, diagnostic methods, prevalence and genetic evolution. Virus Res 226:71-84.

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Article within conference proceedings

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Tran Q, Gao S, Vo NS, Phan V. 2016. Repeat complexity of genomes as a means to predict the performance of short-read aligners. In: BICOB 2016. Proceedings: 2016 Apr 6-4; Las Vegas. Winona (US): International Society for Computers and Their Applications (ISCA). p. 135-42.

Book chapter, or article within a book

Faith DP. 2016. The PD phylogenetic diversity framework: Linking evolutionary history to feature diversity for biodiversity conservation**.** In: Pellens R, Grandcolas P, editors. Biodiversity Conservation and Phylogenetic Systematics. Cham (ZG): Springer International Publishing. p. 39-56.

Complete book

Reddy PP. 2016. Sustainable intensification of crop production. Singapore (SG): Springer Singapore.

Monograph or book in a series

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Book with institutional author

Food and Agriculture Organization. 2016. The State of Food and Agriculture 2016 (SOFA): Climate change, agriculture and food security. Rome (IT): Food and Agriculture Organization of the United Nations.

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Webpage

Michigan State University [Internet]. 2016. Just add water: New discovery in plant-disease mechanism. Rockville, US: ScienceDaily; [updated 2016 Nov 23; cited 2016 Dec 10]. Available from: https://www.sciencedaily.com/releases/2016/11/161123140238.htm

Webpage with Personal Author

Cowie S [Internet]. 2016. 'The Pantanal is national heritage': Protecting the world's largest wetlands. London (UK): The Guardian.com; [updated 2016 Nov 12; cited 2016 Dec22]. Available from: https://www.theguardian.com/global-development-professionals-network/2016/nov/12/pantanal-conservation-wetlands- brazil-paraguay-bolivia

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