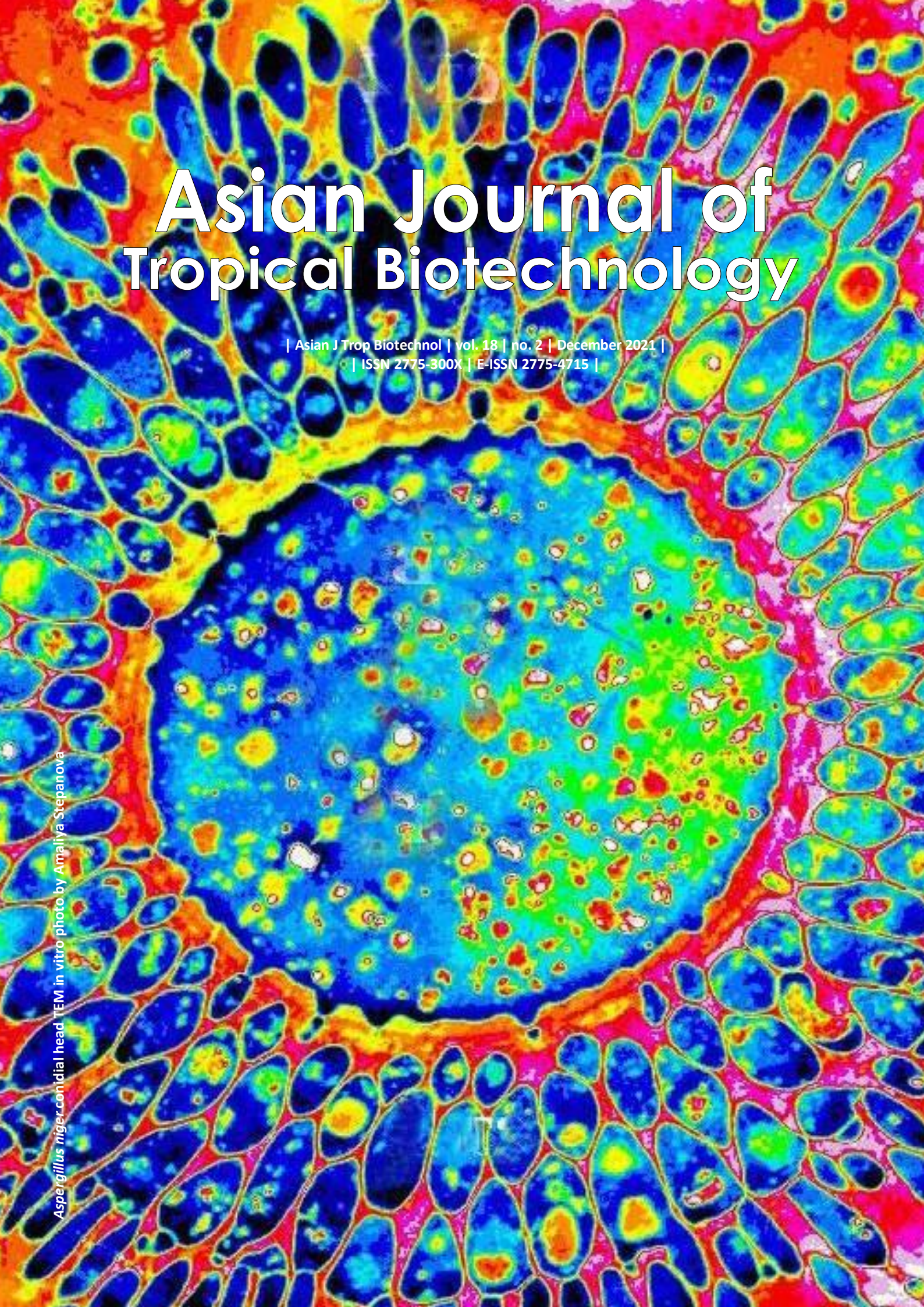
Asian Journal of Tropical Biotechnology

| Asian J Trop Biotechnol | vol. 18 | no. 2 | December 2021 |
| ISSN 2775-300X | E-ISSN 2775-4715 |



Aspergillus niger conidial head TEM in vitro photo by Amaliya Stepanova

Asian Journal of Tropical Biotechnology

| Asian J Trop Biotechnol | vol. 18 | no. 2 | December 2021 |

Published semiannually **PRINTED IN INDONESIA**

ISSN: 2775-300X E-ISSN: 2775-4715

Asian Journal of Tropical Biotechnology

| Asian J Trop Biotechnol | vol. 18 | no. 2 | December 2021|

ONLINE
http://smujo.id/bbs

p-ISSN
2775-300X

e-ISSN
2775-4715

PUBLISHER
Society for Indonesian Biodiversity

CO-PUBLISHER
Universitas Sebelas Maret, Surakarta, Indonesia

OFFICE ADDRESS
Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./Fax.: +62-271-663375, email: editors@smujo.id

PERIOD OF ISSUANCE
June, December

Society for Indonesian Biodiversity

Universitas Sebelas Maret Surakarta, Indonesia

Published by Smujo International for The Society for Indonesian Biodiversity and Sebelas Maret University, Surakarta, Indonesia

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The usage of "et al." in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al. 1999. Future of health insurance. N Engl J Med 965: 325-329. DOI: 10.10007/s002149800025.

Book:

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Chapter in the book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds.). Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.). Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from the internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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ASIAN J TROP BIOTECHNOL
Volume 18, Number 2, December 2021
Pages: 51-54

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c180201

Short Communication: Identification of spoilage fungi in *Myristica fragrans* using DG18 and CYA Media

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Manuscript received: 6 July 2021. Revision accepted: 26 September 2021.

Abstract. *Fendiyanto MH, Satrio RD, Pratami MP, Nikmah IA. 2021. Short Communication: Identification of spoilage fungi in* Myristica fragrans *using DG18 and CYA Media. Asian J Trop Biotechnol 18: 51-54.* To date, very few studies have been conducted on the identification of spoilage fungi in *Myristica fragrans*. Therefore, this study aimed to identify the spoilage fungi in *Myristica fragrans* growing on DG18 media and CYA Identification Media. Isolation of the fungus was carried out by the dilution plating method using a dilution range of 10^{-1} to 10^{-5}. The isolation results at 10^{-2} and 10^{-3} dilutions on nutmeg (*Myristica fragrans*) seed samples indicated the presence of *Aspergillus niger* and *A. flavus*. The fungal species that could be found and identified using the direct plating method were *A. niger*, *A. flavus*, *Penicillium citrinum*, and *Fusarium* sp. The results of fungal isolation showed that the species of spoilage fungi on postharvest of *M. fragrans* seeds, including *Aspergillus flavus*, *A. niger*, *A. tamarii*, *Endomyces fibuliger*, *Eurotium chevalieri*, *E. repens*, and *Penicillium citrinum, Trichoderma* sp. The highest population of spoilage fungi isolated from *Myristica fragrans* seeds was *Aspergillus niger*. This finding indicates that *A.niger* can be identified as spoilage fungi isolated from *Myristica fragrans*.

Keywords: *Aspergillus niger*, *Myristica fragrans*, spoilage fungi

INTRODUCTION

Spoilage fungi could be found in food, particularly in nutmeg (*Myristica fragrans*). Spoilage fungi are fungal species that can destroy organic compounds in many foods like bread, rice, and nutmeg. Spoilage fungi can release mycotoxins into the food to digest organic compounds (Fendiyanto and Satrio 2020). *Aspergillus niger* is one of the spoilage fungi that can release aflatoxin. Aflatoxin can influence human health and can damage the heart, lungs, and vital organs (Fendiyanto and Satrio 2020). Therefore, identification of spoilage fungi in nutmeg is necessary to be conducted using established methods and media.

Fungal isolation media is a medium used to isolate fungi especially molds or yeast. There are many media for isolating and identifying spoilage fungi. The difference between identification media and isolation media is the composition of substances and nutrients contained in them. The types of media classified as media for fungal identification are Czapek Yeast Extract Agar (CYA), Malt Extract Agar (MEA), 25% Glycerol Nitrate Agar (G25N), and Czapek Yeast 20% Sucrose Agar (CY20S). These media are used to identify fungi such as *Aspergillus*, *Penicillium, Trichoderma, Endomyces*, and *Eurotium* in postharvest. The specific medium for identifying *Eurotium* is CY20S media (Pitt et al. 1983). Conversely, the isolation medium used commonly to isolate spoilage fungi or fungi in post-harvest is DG18 media (Fendiyanto et al. 2020). Dichloran 18% Glycerol Agar (DG18) is a medium for isolating xerophilic fungi. Xerophilic fungi are fungi that grow and thrive on substrates with low water content, such as cereals, nuts, flour, nutmeg, and other spices. Meanwhile, the selective or differential isolation medium used to isolate *Aspergillus flavus*, *A. parasiticus*, and *A.nomius* are Aspergillus flavus and Parasiticus Agar (AFPA) media (Pitt et al. 1983; Zummo and Scott 1990; Pitt et al. 1992; Pitt AND Hocking 2009). To suppress *Erwinia carotovora* by in-vitro, fluor-fluorescent *Pseudomonas* was carried out using specific media (Addy 2007)

DG18 media is media that suitable for the growth of fungi species *Eurotium* sp., *Aspergillus* sp., *A. penicillioides*, *Wallemia sebi*, and others (Pitt et al. 1983; Fendiyanto et al. 2020). DG18 media contains chloramphenicol and dichloran. Chloramphenicol is used to inhibit bacteria, while dichloran is used to inhibit the growth of relatively fast-growing fungi such as *Mucor* and *Rhizopus*. However, studies on the use of DG18 and CYA as a medium for the isolation of spoilage fungi on *M. fragrans* were very limited. Therefore, this study aimed to isolate spoilage fungi on *Myristica fragrans* using DG18 Media Culture and identify them using CYA Identification Media.

MATERIALS AND METHODS

Fungal isolation

Fungal isolation was carried out using two methods, namely the direct plating method and the dilution method (Pitt and Hocking 2009). The direct plating method is the semi-quantitative method that is used to isolate the fungi from seeds and determine the seed percentage that is attacked by the fungus. The direct plating method was carried out by placing sterilized nutmeg seeds on DG18 media. We also performed a direct plating method to identify spoilage fungi in the seed of nutmeg. Surface sterilization was performed using 1% of Na-hypochlorite for 2 minutes.

Fungal identification

Pure fungal isolates that grow on DG18 were inoculated on CYA media using inoculation loops aseptically and incubated at 25°C for seven days (Pitt et al. 1992). After incubation, the morphology of the fungi was identified including colony diameter, colony character, and microscopic identification by lactophenol cotton blue wet mount. Colony diameter was determined using a ruler with units of mm. The diameter of the fungus was measured on the reverse side of the petri dish. The observation of colony characters, following the method by Astawan and Kasih (2008) and Hedayati et al. (2007), included colony color, surface texture, and the color of the exudate produced by the fungus.

The composition of DG18 media was firstly reported by Pitt et al. (1983), consisted of glucose 10 g, peptone 5 g, KH2PO4 1 g, MgSO4.7H2O 0.5 g, glycerol A.R. 220 g, agar 15 g, dichloran 2 mg (0.2% in ethanol, 1 ml), chloramphenicol 100 mg, and 1 liter of distilled water. All ingredients except glycerol, chloramphenicol, and dichloran were dissolved in 800 ml of distilled water. After dissolving, distilled water was added until the final volume is 1000 ml. Glycerol was added to obtain a final concentration of 18% w/w. The final pH value of the media was 5.5-5.8. The media was sterilized using an autoclave (Pitt et al. 1983; Pitt et al. 1992; Pitt and Hocking 2009).

The process of making identification media was the same as the process of making fungal isolation media (Aryulina et al. 2005; Ahmad 2005; Fendiyanto et al. 2020). Glassware was sterilized in an oven at 160°C for 2 hours, while identification media were sterilized using an autoclave at 121°C for 15 minutes (Amadi and Adeniyi 2009; Ambarwati et al. 2011).

Enumeration of fungal population

The population of each fungal species in *M. fragrans* was determined based on the number of colonies on the surface of the media after 7 days of incubation. The number of fungal colonies was calculated from the highest to the lowest dilution, namely 10^{-1} to 10^{-5} (Khalimi and Wirya 2008). If the number of colonies in the 10^{-1} dilution can be observed, then the number of colonies is counted on that dilution, and the number of colonies at the lower dilution is ignored. If the number of colonies in the 10^{-1} dilution is too dense to be counted, so the number of colonies is counted at the lower dilution (Saranraj and Geetha 2011). Each species of the fungal colonies counted should be represented in the overall dilution. If the fungal species is not represented in all dilutions, the estimation of the population for that species of fungus is ignored. The fungal population was carried out based on the weight of nutmeg seed (grams) per replication. The fungal population is calculated based on the following formula:

$$\text{Population of each fungus per gram of } M.\ fragrans \text{ per replicate } = \frac{1}{a.b} \times c$$

Where:

a: the volume of the suspension in the cup (1 ml/Petri dish)

b: dilutions that give separate fungal colonies

c: average number of colonies from 3 dishes

Maintenance of fungal isolate

The first stage of maintaining fungal isolates is labeling. Each fungal isolate that was collected was labeled. The label contained the name of the genus or species of fungus, the code of fungal isolate, and the date on which the isolate was rejuvenated. The label was then affixed to the test tube at a distance of + 3 cm below the tube stopper. The second stage was preservation. Preservation is done by pouring sterile paraffin oil into a tube containing 7-day-old fungal isolates 1 cm above the slanted agar. Fungal isolates cultures can survive in paraffin oil for at least 1 year. Fungal isolates in the paraffin oil were stored in the fungal collection room at 20-28°C. Maintenance of fungal isolate culture collections is carried out at least every two years by rejuvenating and re-identifying the isolates.

RESULTS AND DISCUSSION

Fungal isolation by dilution method

Fungal isolation by the direct dilution method is presented in Figure 1. The isolation results at 10^{-2} and 10^{-3} dilutions on nutmeg seed samples indicated the presence of *A. niger* and *A. flavus* fungi (Figure 1). The fungus *A. niger* is characterized by the following characteristics: the color of the colonies is black, the color of the conidium is black, and the hyphae are white, and the surface resembles grass with conidium on it. The fungus *A. flavus* has the characteristics of green colonies in a 10^{-2} dilution and yellowish in a 10^{-3} dilution.

Fungus isolation by the direct plating method

Fungal isolation by direct plating method was carried out by placing nutmeg seeds directly on the isolation media. The direct plating method was carried out following the method by Handewi and Sallem 2002. Five seeds of nutmeg were surface sterilized by soaking in 1% Sodium Hypochlorite for 1 minute and dried them using sterile filter paper. The dried nutmeg seeds were placed on DG18 standard media and incubated for 7 days (Figure 2.)

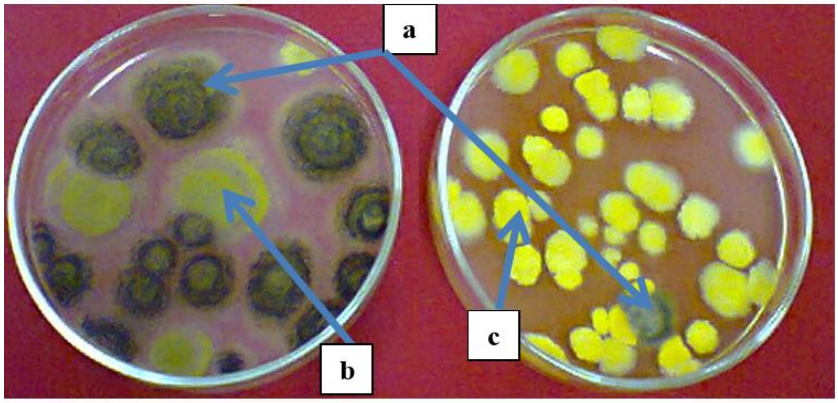

Figure 1. Fungal isolation by dilution method on CYA Media. Left 1:100 dilution, right 1:1000; *Aspergillus flavus* (b), *A. niger* (a), and *Eurotium repens* (c).

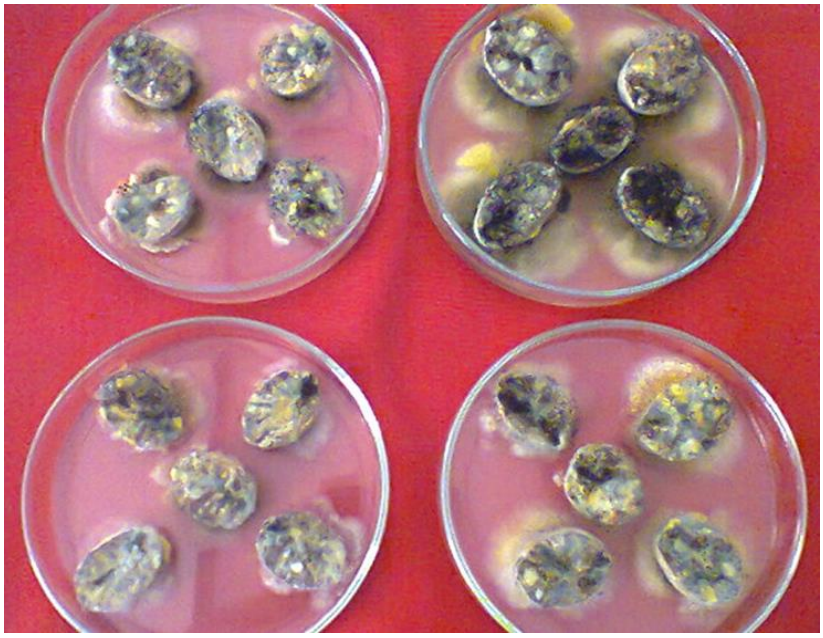

Figure 2. Fungal isolation by the direct plating method on DG18 media.

Figure 2 showed that fungal colonies were emerging from the nutmeg seed samples. The species of fungi that emerged from nutmeg seeds include *A. niger*, *A. flavus*, *Penicillium citrinum*, and *Fusarium* sp. The highest fungal population that attacks nutmeg seeds was *A. niger* (100%), while the lowest population was *Penicillium citrinum* (25%).

Enumeration of fungal population

The fungal population was calculated based on the number of colonies in a certain dilution. The fungal population from the nutmeg seed sample was presented in Table 1. The fungal species from the nutmeg seed that can be identified are the fungal species with specific characteristics of colony color and surface texture. These species of fungi were identified using an identification book based on colony diameter after 7 days of incubation and the colony color on standard media (Pitt and Hocking 2009). These findings are similar to Gibson et al. (1994) in predicting the effect of water activity on fungal growth of *Aspergillus flavus* and related species. These findings are also similar to Fendiyanto et al. (2020) in identifying spoilage fungi in bread.

The highest fungal population that emerged from nutmeg seeds was *Aspergillus niger* (Table 1). *A. niger* is characterized by black conidium with a diameter of 8 to 9 cm on CYA standard media (Pitt and Hocking 2009). The high population of *A. niger* due to the conidium of *A. niger* is easy to fly and grow on standard media compared to other species of fungi. The lowest fungal populations were *Eurotium chevalieri* and *E. repens* (Table 1).

Table 1. The fungal population on nutmeg seed samples in the first month of storage.

Sample code	Weight (g)	Fungi	Replications			Dilution factor
			1	2	3	
NUT100	25.00	*Aspergillus flavus*	5	5	4	10^2
		A. niger	8	7	6	10^3
		A. tamarii	1	2	2	10^3
		Endomyces fibuliger	2	0	2	10^3
		Trichoderma sp.	3	3	8	10^3
NUT200	25.01	*A. flavus*	5	5	9	10^2
		A. niger	3	11	6	10^2
		A. tamarii	12	12	11	10^2
NUT300	25.01	*A. flavus*	2	3	3	10^3
		A. niger	1	4	2	10^3
		A. tamarii	2	2	1	10^2
		E. fibuliger	4	3	0	10^3
NUT400	25.00	*A. flavus*	13	11	10	10^2
		A. niger	11	11	7	10^2
		A. tamarii	7	1	5	10^2
		E. fibuliger	5	7	6	10^3
NUT500	25.00	*A. flavus*	3	5	0	10^2
		A. niger	22	17	13	10^2
		A. tamarii	2	3	2	10^2
		E. fibuliger	1	3	4	10^3
		Eurotium chevalieri	1	1	4	10^2
		E. repens	1	2	3	10^2
NUT600	25.01	*A. flavus*	5	6	9	10^1
		A. niger	9	10	5	10^2
		A. tamarii	1	1	8	10^2
		E. fibuliger	4	5	4	10^2

In addition, we can classify the potency of many fungi as biocontrol agents soon, i.e., understanding antagonistic agent (Fernando et al. 2005; Fendiyanto and Satrio 2020), identification of potential metabolites (Fendiyanto et al. 2020; Fendiyanto et al. 2021), and finding the genetic architecture of the organism (Fendiyanto et al. 2019a; Fendiyanto et al. 2019b; Satrio et al. 2019; Pratami et al. 2020).

In conclusion, the results of fungal isolation showed that spoilage fungi found in postharvest of nutmeg seeds were *Aspergillus flavus*, *A. niger*, *A. tamarii*, *Endomyces fibuliger*, *Eurotium chevalieri*, *E. repens*, and *Penicillium citrinum*, *Trichoderma* sp. The highest population of fungi isolated from *Myristica fragrans* seeds was *Aspergillus niger*.

ACKNOWLEDGEMENTS

This research was partially supported by the Ministry of Research, Technology, and Higher Education through Dr. Miftahul Huda Fendiyanto, and Dr.(c) Rizky Dwi Satrio, in the fiscal year of 2014-2015 through PKM research grant. We thank the Laboratory of Phytopathology, SEAMEO BIOTROP, Bogor, West Java. We thank Ina Retnowati for their guidance in an antagonistic test of spoilage fungi against *A. niger*.

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ASIAN J TROP BIOTECHNOL
Volume 18, Number 2, December 2021
Pages: 55-68

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c180202

Genetic transformation of banana with Extracellular Secreted *Plant ferredoxin-like protein* (ES-*Pflp*) gene

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Manuscript received: 17 August 2021. Revision accepted: 14 October 2021.

Abstract. *Wanjiku SM, Runo S, Tripathi L. 2021. Genetic transformation of banana with Extracellular Secreted* Plant ferredoxin-like protein *(ES-*Pflp*) gene. Asian J Trop Biotechnol 18: 55-68.* Banana *Xanthomonas* Wilt (BXW) is the most damaging disease of banana (*Musa* spp.) in East Africa. It is caused by *Xanthomonas campestris* pv. *musacearum* (Xcm). This disease has no effective control strategies. All banana cultivars are susceptible to BXW disease. Due to their sterility and long generation life cycle, most cultivated cultivars are triploids, hence difficult to enhance through conventional breeding. Genetic engineering provides an alternative and successful method of BXW disease prevention. The purpose of this work was to express the Extracellular Secreted *Plant ferredoxin-like protein* (ES-*Pflp*) gene in bananas and to assess the resistance of transgenic lines to Xcm. A signal peptide guides the *Pflp* to the extracellular area of the cell. In *Arabidopsis*, ES-*Pflp* has been found to provide bacterial resistance. Thus, overexpression of ES-*Pflp* in a banana is expected to boost resistance to Xcm. Co-cultivation of Gross Michel and Sukali Ndizzi Embryogenic Cell Suspensions (ECSs) with *Agrobacterium* strain EHA105 carrying the binary vector pBI-ES-*Pflp*, followed by the selection of kanamycin-resistant calli and regeneration of plantlets. Transgenic banana plants producing an ES-*Pflp* gene were produced using the constitutive promoter of the Cauliflower mosaic virus 35S. Polymerized Chain Reaction (PCR), Southern blot hybridization, and Reverse Transcription Polymerized Chain Reaction (RT-PCR) studies of transgenic lines demonstrated sustained transgene incorporation and expression. Growth research shows that most transgenic lines grew similarly to non-transgenic plants under glasshouse conditions and thus were proceeded to screening for resistance to BXW disease. Agronomic data were collected on all screened lines and compared to control lines. Transgenic lines were screened for increased resistance to BXW disease by artificial inoculation with Xcm. Developing banana cultivars resistant to BXW would increase banana production and help farmers who rely on bananas as a staple food and income crops maintain food security.

Keywords: Banana *Xanthomonas* Wilt, disease, ES-*Pflp*, genetic, *Xanthomonas campestris* pv. *musacearum*

Abbreviations: BRM: Bacterial Resuspension Medium, BBTV: Banana Bunchy-Top Virus, BSV Banana Streak Virus, BXW: Banana *Xanthomonas* Wilt, ECSs: Embryogenic Cell Suspensions, ES-*Pflp*: Extracellular Secreted *Plant ferredoxin-like protein*, HR: Hypersensitive Response, PCR Polymerized Chain Reaction, RT-PCR: Reverse Transcription Polymerized Chain Reaction, SAR: Systemic Acquired Resistance, Xcm: *Xanthomonas campestris* pv. *musacearum*

INTRODUCTION

Bananas and plantains are the world's sixth-largest agricultural crop, trailing maize, rice, wheat, potato, and cassava, with an estimated annual global production of 139 million tons (FAOSTAT 2012). A third of this is produced in Africa, with East Africa producing and consuming the most. India is the world's largest producer of bananas, followed by Uganda, which has 24.7 million tons and 9.77 million tons sequentially (FAOSTAT 2012). Over 130 countries in the tropics and subtropics farm this crop (Pachuau et al. 2014), making it the most domesticated clonal crop (De Langhe et al. 2010). Bananas are a year-round crop (Namuddu et al. 2012). They can grow on steep slopes (Karamura et al. 1998) providing a staple food source (Hasanah et al. 2017) and source of income for millions of people, particularly in Africa, with approximately 87% of production remaining in domestic markets (Roux et al. 2008).

Numerous diseases and pests adversely affect banana production, including black sigatoka (*Mycosphaerella fijiensisi*), Banana *Xanthomonas* Wilt (BXW) caused by *Xanthomonas campestris* pv. *musacearum* (Xcm), *Fusarium* Wilt (*Fusarium oxysporum f.* sp. *Cubense*), viruses [Banana Bunchy-Top Virus (BBTV), genus *Nanavirus* and Banana Streak Virus (BSV), genus *Badnavirus*], weevils and nematodes (Jones 2000; Tushemereirwe et al. 2004; Tripathi et al. 2008; Hadiwiyono 2011). Given the banana's global importance, there is considerable opportunity for developing disease-free and high-yielding cultivars for growers. Resistant cultivars would be the most cost-effective and environmentally friendly way to overcome these impediments. Conventional breeding has had limited success in improving banana genetics due to long generation durations, a lack of genetic variety, an undesirable gene pool, and time-consuming screening methods (Namuddu et al. 2012), and the triploidy of many farmed bananas (Vuylsteke 2000). Therefore, while a few

diploid clones produce viable pollen, most of the commercial clones' germplasm is both male and female-sterile (Novak et al. 1989). Genetic engineering is a possible alternative technique for creating superior agronomic features in bananas and plantains.

Plants have many defense systems in place to stave against disease invasion. The Hypersensitive Response (HR) is an induced resistance mechanism characterized by fast, localized cell death in response to infection by a microbial pathogen (Goodman and Novacky 1994; Dangl et al. 1996). HR-induced cell death creates a physical barrier that prevents further pathogen infection. Additionally, a local HR is frequently accompanied with the activation of plant defense responses in adjacent and even distal uninfected plant sections, resulting in the establishment of Systemic Acquired Resistance (SAR). Human resistance is frequently observed in disease-resistant plants. It often occurs before a slower systemic (whole plant) response, resulting in SAR (Freeman 2003). It has been demonstrated that the *Plant ferredoxin-like protein* (*Pflp*) activates HR, which is characterized by planned cell death in response to microbial infection. It has been shown that constitutive expression of the *Pflp* gene in transgenic tobacco, *Arabidopsis*, and banana plants confers increased resistance to various diseases (Huang et al. 2004; Lin et al. 2010; Namukwaya et al. 2012). Additionally, it was discovered that extracellular *Pflp* released by the cell increased resistance to *Ralstonia solanacearum* in *Arabidopsis* (Huang et al. 2004). As a result, the purpose of this study was to convert bananas with Extracellular Secreted *Plant ferredoxin-like protein* (ES-*Pflp*) and determine the amount of resistance to BXW of transgenic lines.

Banana (*Musa* spp.) is a major global food crop grown in over 130 tropical and subtropical nations. However, BXW disease has had a significant impact on banana output in Eastern Africa, jeopardizing the livelihoods of millions of farmers (Tushemereirwe et al. 2003). BXW is a highly damaging disease that affects all banana kinds, including dessert, frying, roasting, and beer variants (Ssekiwoko et al. 2006). The effects of BXW disease are severe and quick, in contrast to the impact of other diseases, which induce progressive increases in mortality over the years. The economic consequence of BXW disease includes yield loss in absolute terms and the mortality of mother plants that would otherwise contribute to ratoon plant production cycles (Tripathi et al. 2009). Over a decade, economic losses are anticipated to total $2 billion due to price rises and considerable production decreases (Abele and Pillay 2007). Due to the lack of known natural sources of resistance in any cultivated banana, genetic modification is widely regarded as the most viable strategy for developing bacterial wilt-resistant bananas, especially given the availability of an efficient and reliable banana transformation protocol (Tripathi et al. 2012). As such, the purpose of this work was to determine the possibility of adopting genetic engineering approaches to reduce BXW sickness by utilizing the ES-*Pflp* gene.

The specific objectives of this study were: i) to transform and create transgenic banana cultivars Gross Michel and Sukali Ndizzi with ES-*Pflp* gene; ii) to determine the effect of overexpression of ES-*Pflp* gene on resistance to BXW disease in transgenic Gross Michel and Sukali Ndizzi plants grown in glasshouse conditions; iii) to compare the degrees of BXW resistance in Gros Michel ES-*Pflp* and Gros Michel *Pflp* transgenic lines.

MATERIALS AND METHODS

Plant materials and explant preparation for transformation experiments

IITA donated Embryogenic Cell Suspensions (ECSs) of the banana cultivars Sukali Ndizzi and Gros Michel for this study. Tripathi et al. (2010; 2012) outlined the process for the subculture of ECSs. Sukali Ndizzi ECSs were subcultured in MA2 medium. In contrast, Gros Michel ECSs were subcultured in ZZ medium [A settled cell volume of 1 mL of ESCs was subcultured in 50 mL of their respective media for 5 days before transformation to maximize cell transformation competence and efficiency (Tripathi et al. 2012).

Agrobacterium and plasmid

The pBI-ES-*Pflp* vector was utilized in this research. It contained the ES-*Pflp* gene driven by the CaMV35S promoter and the nptII gene as a selection marker (Figure 1). The International Institute of Tropical Agriculture purchased this construct from Academia Sinica in Taiwan via the African Agricultural Technology Foundation. The vector-bearing *Agrobacterium* strain EHA105 was grown on LB medium supplemented with kanamycin (50 mg/L) and rifampicin (50 mg/L). To prepare for transformation, the bacterial culture was cultivated for two days at 28°C with shaking in liquid LB medium supplemented with the required antibiotics. A day before transformation, approximately 500 mL of the *Agrobacterium* culture was added to 20 mL of LB medium containing suitable antibiotics and then cultured overnight at 28°C in a shaking incubator (200 rpm). On the 21st day after transformation, the bacteria were harvested by centrifuging at 5,000 rpm for 10 minutes at 4°C, and the pellet was re-suspended in 25 mL of Bacterial Resuspension Medium (BRM) (Tripathi et al. 2012) and shaken at 70 rpm for 2 hours at 25°C for induction. The optical density of the culture at 600 nm was determined and adjusted to 0.6 using BRM media.

Transformation, selection, and regeneration of transgenic plants

In the transformation studies, ECSs of Sukali Ndizzi and Gros Michel's banana cultivars were employed as explants. They transferred the ECSs into falcon tubes and allowed the cells to settle. Excess media was discarded. Ten milliliters of the appropriate culturing media were added, warmed to 45°C, mixed, and incubated for 5 minutes. The medium was decanted and 110 µL of 2% pluronic acid and the pre-induced *Agrobacterium* suspension were added to ECSs. Next, the mixture was centrifuged at 900 rpm for 6 minutes at 25°C and the contents of the tube left tosettle. After that, the cells were

allowed to stand for 30 minutes for infection. The liquid was then decanted, and agro-infect cells were co-cultured for 3 days in the dark on a bacterial co-culture medium (Tripathi et al. 2012).

Following co-cultivation, the Agro-infected ECSs were washed three times with MA2 for Sukali Ndizzi and ZZ liquid for Gros Michel, supplemented with 300 mg/L cefotaxime, i.e., after plating the cells on a sterile nylon mesh, they were transferred to MA3 medium with 300 mg/L cefotaxime and 100 mg/L kanamycin and maintained at 26°C in the dark. Every 14 days, the cells on the sterile mesh were transferred to fresh media. After two to three months of selection, embryos were moved to RD1 medium supplemented with 300 mg/L cefotaxime and 100 mg/L kanamycin and housed at 26ºC in the dark. After germination in the dark at 26ºC, mature embryos were transferred to MA4 medium supplemented with 300 mg/L cefotaxime and 100 mg/L kanamycin. Sukali Ndizzi germinated plantlets were placed to proliferation media, while Gros Michel germinated plantlets were transferred to the basal medium.

DNA extraction and Polymerized Chain Reaction (PCR) analysis of the regenerated plants

Using the DNeasy plant micro kit, genomic DNA was extracted from thirty lines of Gros Michel and 24 lines of Sukali Ndizzi putative altered plantlets (Qiagen, GmbH, Germany). The presence of the ES-*Pflp* gene in the genomes of randomly selected putatively transgenic lines was confirmed using Polymerized Chain Reaction (PCR) analysis using gene-specific primers having a predicted band size of 450 base pairs. *Pflp* gene primers were 50 CAAGAAAACCAGCTGTGACAAGCCTTAAAC 30 and 50 CGAGTTCTGCCTCTTTGTGAGTCTCAATAG 30; nptII gene primers were 50 CCTATCCGCAACTTCTTTACCTA 30 and reverse 50 ACACCCAGCCGGCCACAGTCG 30. PCR reactions were carried out using an Eppendorf Master Cycler (EPAG 5341 012727, H Hamburg, Germany). 2l plant DNA, 1mM MgCl2, 0.4M primers, 2.5X PCR buffer, 0.75M dNTPs, and 0.001 units Taq Hot star in a 25l reaction volume. This reaction volume was initially denaturated at 94ºC for 10 minutes, followed by 35 cycles of denaturation at 94ºC for 45 seconds, annealing at 58ºC for 1.3 minutes, extension at 72ºC for 1 minute, and final extension at 72ºC for 7 minutes. The positive control was pBI-ES-*Pflp* plasmid DNA, while the negative was non-transgenic plant DNA and water. Electrophoresis of the PCR products in a 0.8 % l agarose gel in tris base acetic acid EDTA buffer containing 2l gel red was performed. The results were seen using a UV transilluminator.

Multiplication, rooting and acclimatization of transgenic plants

Each of the PCR-positive transgenic lines were doubled to get at least six duplicates. Two duplicates of each line were retained as the mother plant culture, while the remainders were planted in the rooting medium. After washing the rooted plants to remove any remaining media, they were placed in plastic cups containing sterile soil in a 1:1 ratio with manure. They were hardened off for a month in a plastic chamber in the glasshouse and then transferred to larger pots (15 cm diameter) for continued growth. Three-month-old plants were utilized to screen for BXW resistance.

Screening of transgenic lines for resistance to BXW disease

An Xcm culture was grown at 28ºC on YPGA-CC media with cephalexin (50 mg/L) and cycloheximide (200 mg/L) for two days. The culture's OD was determined and centrifuged to settle the cells. Then the media was drained out. The pellet was re-suspended in sterile water and the optical density (OD600nm) was set to 1.0. The suspension was prepared for inoculation by placing it in 1.5 mL tubes. The second open leaf of each three-month-old line in the glasshouse was infected with 100 µl of Xcm culture using a hypodermic needle. For 60 days, the inoculated plants were housed in a glasshouse and monitored for disease symptoms. The disease severity of the inoculated plants was determined using a 0-5 scale: 0 no symptoms, 1 only the inoculated leaf wilted, 2-2 to 3 leaves wilted, 3-4 to 5 leaves wilted, 4 all leaves wilted, but the plant remained alive, and 5 the entire plant died. The percentage of resistance was estimated as (Reduction in wilting in comparison to control plants/Total number of wilted leaves in control plants) X 100.

Genomic DNA isolation and Southern blot analysis

Southern hybridization was used to examine the ES-*Pflp* gene's incorporation into the banana genome. In this study, it was done utilizing the DIG labeling and detection system.

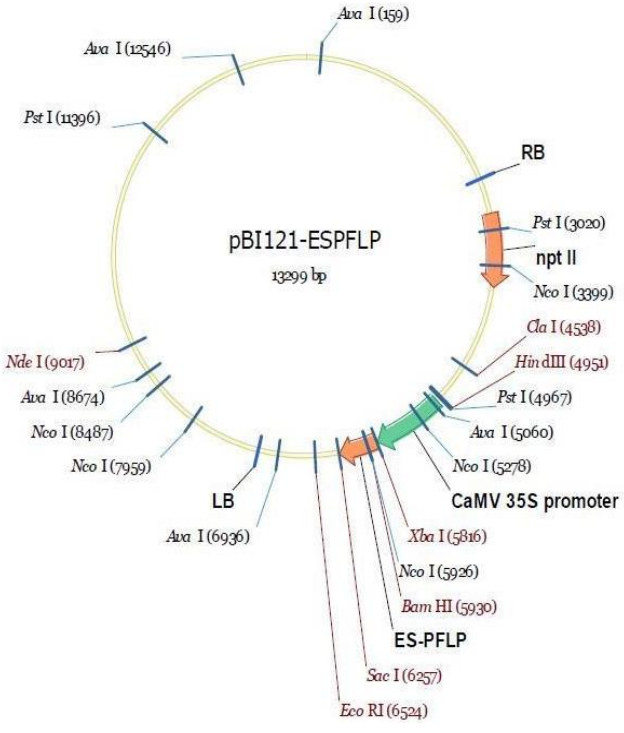

Figure 1. Map of pBI 121-ES-*Pflp* plasmid vector (Lin et al. 2011).

Genomic DNA isolation

Using the hexadecyltrimethylammonium bromide (CTAB) technique, genomic DNA was extracted from leaf tissue of six Sukali Ndizzi ES-*Pflp* transgenic lines and six Gros Michel ES-*Pflp* transgenic lines that demonstrated significant resistance during glasshouse screening and a non-transgenic plant (Gawel and Jarret 1991). Around 1 gram of leaf tissue was crushed in liquid nitrogen and incubated for 30 minutes at 65 °C in 700 µl of extraction buffer (2% CTAB, 200 mM Tris-HCl [pH 8], 0.14 M NaCl, 0.1% mercaptoethanol, and 20 mM EDTA).

Total DNA was extracted with 700 µl chloroform-isoamylalcohol (24:1) v/v and precipitated with the same amount of isopropanol. After washing with 1 mL of 70% cold ethanol, the DNA was treated with RNAse A, re-extracted, re-precipitated, washed, and re-suspended in sterile water. Southern analysis was performed on the measured DNA.

Preparation of probe

The PCR DIG probe synthesis kit was used to synthesize the DIG-labeled probe. one µl of Taq polymerase, 5 µl dNTP mix, 1 µl of plasmid DNA, and 0.5 µM of forward and reverse primers each were used in the 50 µl PCR reactions. Denaturation at 95ºC for 2 minutes was followed by 30 cycles of 94ºC for 30 seconds, followed by temperatures of 55ºC for 30 seconds, 72ºC for 2 minutes, and a final extension at 72ºC for 5 minutes.

Restriction analysis of genomic DNA

According to Table 1, the Hind *III* restriction enzyme was used to digest the plant genome.

After overnight incubation at 37ºC, the mixture was lyophilized to concentrate to a volume of 30 µL. On a 0.8% gel, the limited DNA was resolved. Positive control was plasmid DNA, and negative control was a non-transgenic plant. The gel was first operated at 60V for 30 minutes and then at 50V for 6 hours. GeneSys gel documentation system was used to capture the image. The gel was then divided into 10 cm by 10 cm squares, making tiny gels more manageable and processable.

Preparation of the gel for Southern blotting

Depurination. Restricted DNA on the gel was broken to aid membrane transfer, as DNA more extended than 10 kb does not transfer. This was accomplished through acid depurination. The gel was immersed in a 0.25 M HCl solution and gently shook for 15 minutes. Bromophenol blue's color should change from blue to yellow to indicate that the gel has been saturated with acid. After that, the gel was gently rinsed with sterile distilled water.

Table 1. DNA restriction reaction.

DNA (10 µg)	40 µL
Restriction enzyme (Hind *III)*	5 µL
Sterile water	45 µL
Buffer 10X	10 µL
Final volume	100 µL

Denaturation. The double-stranded DNA was denatured by incubating the gel in a denaturation buffer (200 mL) for 30 minutes at 50 rpm while shaking it constantly. By the end of the incubation period, the bromophenol blue in the gel had transformed from a yellow to a blue color.

Neutralization. The denaturation buffer was discarded, and the gel was immersed in neutralizing buffer for 30 minutes while gently shaking throughout the procedure.

Blotting of gel. The gel was wrapped in parafilm and placed more extensively than the 10X saline sodium citrate tray. Two lengths of Whatman 3 MM paper were cut more comprehensive than the gel and quickly soaked in 10X SSC, before being placed in a glass plate. Air bubbles were eliminated between the paper and support by rolling the pipette back and forth across the surface many times. One sheet of blotting paper and four sheets of Whatman 3 MM paper were cut to be approximately 1mm more prominent on each side than the gel. The membrane was handled with gloves and with blunt-end forceps at the edges. On the platform, the prepared gel was placed upside down. Rolling a 1 mL pipette tip back and forth over the gel freed trapped air bubbles. The gel was then encased in plastic wrap to ensure that the 10X SSC flows through it rather than around it. Next, the precut nylon membrane was placed on the gel and air bubbles were eliminated between the gel and the blotting paper. Four pre-cut sheets of Whatman 3MM paper were moistened in 2X SSC, placed on the nylon membrane, and then trapped air bubbles were removed. On top of the Whatman filter sheets, 20 cm of dry paper towels were laid. This ensures that the plastic wrap enclosing the gel prevents contact between the paper towels and the 10X SSC and moist filter paper behind the gel. It was assured that the towels did not fall over the side, as this could result in the liquid flowing around the gel rather than through it. On top of the paper towels, a second glass top was installed, and a weight was placed on top of the plate. The transfer was permitted to continue for twenty hours, while wet paper towels were replaced with dry ones to guarantee efficiency.

Fixing the DNA to the blot. Following completion of the transfer, the weight, paper towels, and four sheets of filter paper were removed. Next, the gel was overturned with the nylon membrane and laid them gel side up on moist filter paper. To ensure that the gel lanes could be identified afterwards, the positions of the gel lanes on the membrane were marked with a soft lead pencil. After that, the gel was peeled away from the membrane. After staining the gel with gel red, the efficiency of DNA transfer was determined. Finally, the DNA was fixed to the membrane using UV cross linking at a power of 12X 1000V. This led to increased accuracy and sensitivity.

Pre-hybridization and hybridization. Dig hybridization buffer (20 mL) was added to a clean hybridization bottle with the membrane facing the buffer rather than the bottle wall. This was incubated at 42°C for 3 hours at a speed of 60 rotations per minute. After replacing the solution with 15 mL DIG hybridization buffer containing PCR labeled probe, the mixture was incubated overnight at 42°C and 60 rpm.

Stringent washes and detection. For the high salt wash, the membrane was washed twice in 200 mL sterile water containing 2X SSC and 0.1 percent sodium dodecyl sulfate (w/v) for 5 minutes and shaken on an orbital shaker. This was followed by a low salt wash at 65ºC in a preheated 0.5 X SSC and 0.1 % SDS (w/v) buffer for 15 minutes twice. Each wash contained 200 mL buffer. For 5 minutes, the membrane was normalized in 20 mL washing buffer (100 mM maleic acid and 0.3% tween 20). After that, the membrane was immersed in 70 mL blocking solution. Three µL of antibodies (AP conjugate) in a 1: 10000 dilution was added to 30 mL of the remaining blocking solution and used to wash the membrane at 60 rpm. After that, the membrane was washed twice with 100 mL washing buffer at 60 rpm. For 5 minutes, the membrane was incubated with 20 mL detection buffer. After that, the membrane was placed on cling film with the DNA side facing up and prepared for usage. Add disodium 3-(4-methoxyspiro 1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.13,7] decan-4-yl) phenyl phosphate (CSPD) and cover with cling film. Excess CSPD was eliminated after 5 minutes. In the dark, an X-ray film was inserted in an X-ray film holder and the membrane was transferred to it. The X-ray film was produced as follows: 5 minutes in developer solution, 30 seconds in distilled water, and 5 minutes in fixer solution to visualize the X-ray film. This was done in a dimly lit room.

RNA extraction and RT- PCR

The lines that demonstrated resistance to BXW disease screening in the glasshouse (six of Gros Michel and six of Sukali Ndizzi) were Reverse Transcriptase PCR (RT-PCR) tested for expression of the desired gene. A leaf tissue sample (100 mg) was pounded in liquid nitrogen using a motor and pestle treated with Diethylpyrocarbonate, and total RNA was extracted using the Qiagen RNeasy Kit (Qiagen, Maryland, USA) (Qiagen 2010). To avoid DNA contamination, the isolated RNA was processed with DNase. Quantification and normalization of the isolated RNA were performed. The whole RNA template (1 g) was utilized to synthesize cDNA in a 20:l reaction mix, using the first-strand cDNA synthesis procedure from thermo scientific. This reaction contained 13 liters of RNA, 1 liter of oligo (dT) 18 primer, 1 liter of 10 mM dNTP mix, 4 liters of 5X RT Buffer, 1 liter of Maxima H Minus Enzyme Mix, and nuclease-free water. The reaction mixture was gently stirred and then incubated at 50ºC for 30 minutes. The reaction was brought to a halt by heating to 85ºC for 5 minutes. The cDNA generated was utilized for RT-PCR.

The PCR reaction was carried out using 100 mg of cDNA synthesized. In a 25 µL reaction volume, this reaction contained 1 mM MgCl2, 0.4 µM of each primer, 2.5X PCR buffer, 0.75 µM dNTPs, and 0.001 units Taq Hot star. This reaction volume was initially denatured for 10 minutes at 94ºC, followed by 35 cycles of denaturation at 94ºC for 45 seconds, annealing at 55ºC for 1 minute 30 seconds, extension at 72ºC for 1 minute, and final extension at 72ºC for 7 minutes.

Positive controls included pBI-ES-*Pflp* plasmid DNA, whereas negative controls included non-transgenic plant DNA and water. After electrophoresis, , the PCR products were separated and observed using a UV transilluminator in a 0.8 percent agarose (w/v) gel stained with 2l gel red.

Measurement of plant growth

The growth of the transgenic lines for each cultivar was measured at 90 days post-weaning and compared to the growth of the control non-transgenic plant at the same time. The physiological characteristics that were measured were leaf length, leaf number, pseudostem base diameter, and plant height, amongst others.

Data analysis

The studies were carried out in a completely randomized design, and each experiment was carried out in three different replicates to ensure accuracy. As a proportion of the number of independent lines per milliliter of settling cells volume, transformation efficiency was measured for each experiment. One-way ANOVA (software GenStat 14 edition) was used to evaluate the data, and Duncan's Multiple Range Test was used to compare the differences between the groups.

RESULTS AND DISCUSSION

Transformation of cell suspension and regeneration of transgenic plants

Gros Michel and Sukali Ndizzi embryogenic cell suspensions transformed with ES-*Pflp* were effectively selected for embryo initiation, maturation, and development on 100 mg/L Kanamycin-containing MA3, RD1, and MA4 medium, respectively. Every two weeks, cells or embryos were transferred to fresh media with 100 mg/L Kanamycin and 300 mg/L Cefotaxime for selection. Sukali Ndizzi ECSs were first implanted in MA3 medium with proper antibiotics until tiny white embryos were regenerated in three weeks and Gros Michel ECSs in four weeks. Sukali Ndizzi embryos were then placed in RD1 medium with suitable antibiotics for one month and Gros Michel embryos were placed in RD1 medium for six weeks for embryo development. The developing embryos were then placed in MA4 media supplemented with antibiotics to mature into plantlets. All these procedures were carried out in complete darkness. Following plantlet production, plates of MA4 (Figures 2 and 3) were exposed to light for one week before being placed in appropriate proliferation conditions for growth and multiplication. For both cultivars, approximately one hundred putatively altered lines were created.

As selection progressed, a substantial difference between the transformed and non-transformed cells became apparent, with the latter becoming brown to black and eventually dying. In contrast, the transgenic cells continued to multiply (Figure 2).

Transformation efficiency

For each cultivar, the efficiency of embryo regeneration to plantlets following transformation was determined by doing three transformation experiments with a settling cell

volume of 1 mL each. As shown in Figure 4, Sukali Ndizzi had greater embryo regeneration efficiency than Gros Michel. This demonstrates that the Sukali Ndizzi cultivar was more efficient at transformation than the Gros Michel cultivar.

Molecular characterization of transgenic plants

Polymerized Chain Reaction

The PCR analysis employing *Pflp* specific primers on randomly selected putative transgenic plantlets (31 lines of Gros Michel and 24 lines of Sukali Ndizzi) confirmed the presence of a 450 bp band in the PCR profiles (Figure 5 A, B, C and D). In addition, the existence of the banana housekeeping gene was confirmed using the 25S housekeeping gene in PCR positive lines of both cultivars, and an expected band size of 110 bp was detected (Figure 5).

RT-PCR analysis

In this study, PCR positive lines of both cultivars Gros Michel and Sukali Ndizzi were randomly selected and subjected to reverse transcription polymerized chain reaction analysis. The ES-*Pflp* primers with an anticipated band size of 178 bp were used to achieve this result, as depicted in Figure 6. This was done to evaluate the transgene expression in the lines that had been chosen.

Southern blot analysis

Selected Sukali Ndizzi ES-*Pflp* lines were assessed for stable integration of the ES-*Pflp* transgene using *Pflp* gene-specific probe, as shown in Figure 7.

Acclimatization of transgenic plants

A total of 18 Sukali Ndizzi lines and 30 Gros Michel lines were rooted and weaned in the soil after PCR positive. All the lines were toughened and then potted for a total of two months before being screened (Figure 8).

Growth analysis of banana plants to be screened

Growth parameters of Gros Michel ES-Pflp lines

When comparing the 90-day old potted transgenic lines for each cultivar to the control line, growth parameters such as leaf number, leaf length, pseudostem width, and plant height were measured on the transgenic lines (Figure 9).

When compared to the control line, the pseudostem width of all line metrics revealed no significant difference; $p=0.0847642$, where $\alpha=0.05$. However, the height of the plants on line numbers 72, 27 and 77 differed significantly from the height of the control line. Lines 72 and 77 had considerably higher plant heights when compared to the control line, while line 27 had substantially lower plant heights when compared to the control line; $p=0.0295013$ when 0.05 was used as the significance threshold. This is seen in Figure 10.

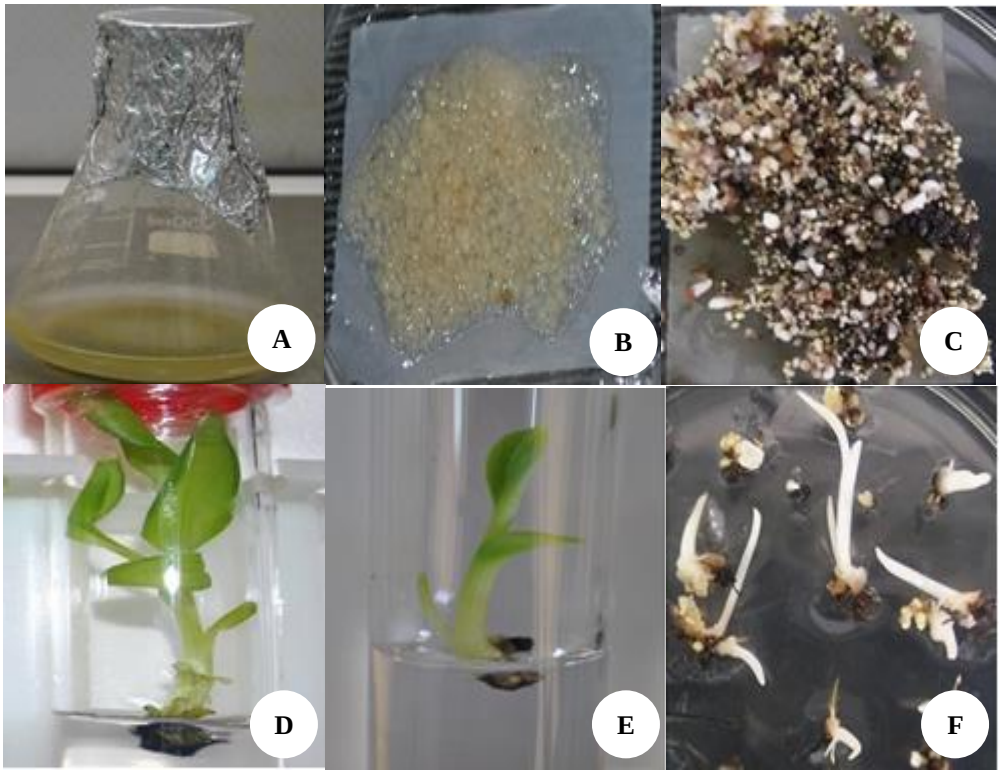

Figure 2. Transformation, selection, and regeneration of transgenic plants of Sukali Ndizzi. A: Embryogenic cell suspension, B: Agro-infected cells, C: Selection of cells on embryo development media (MA3), D: Embryos on selection on RD1 medium, E and F: Shoots in proliferation media.

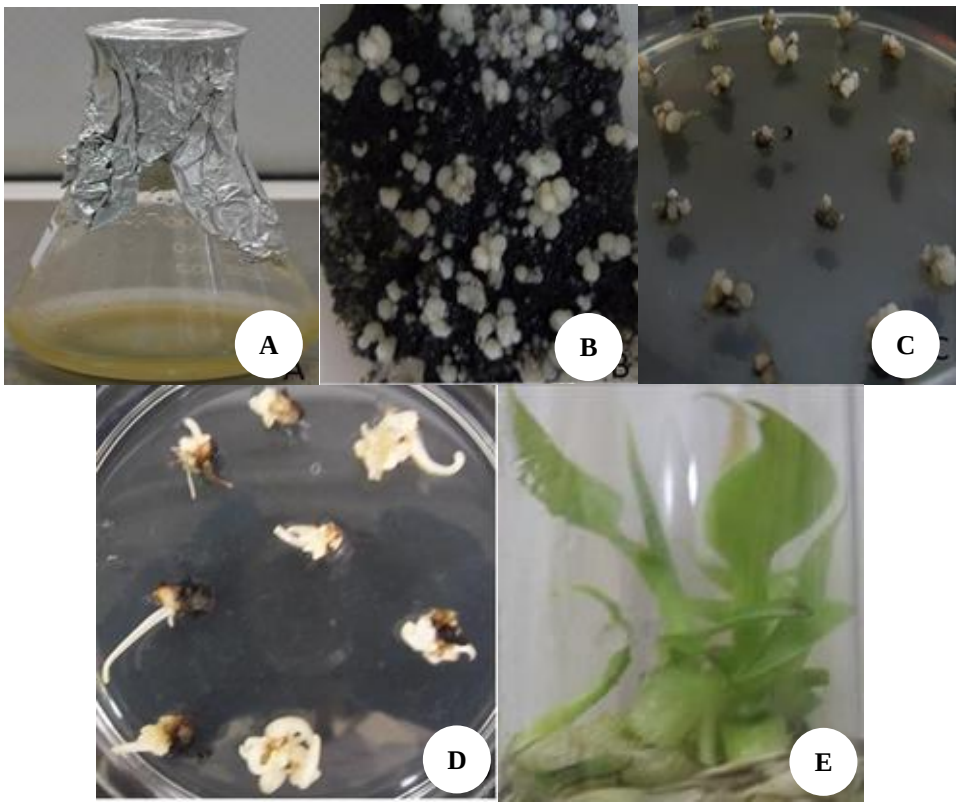

Figure 3. Transformation, selection, and regeneration of transgenic plants of *Gros Michel*. A: Embryogenic cell suspension, B: Selection of cells on embryo development media (MA3), C: Embryos on selection in development media (RD1), D: Embryos on selection in maturation media (MA4), E: Shoot inproliferation media.

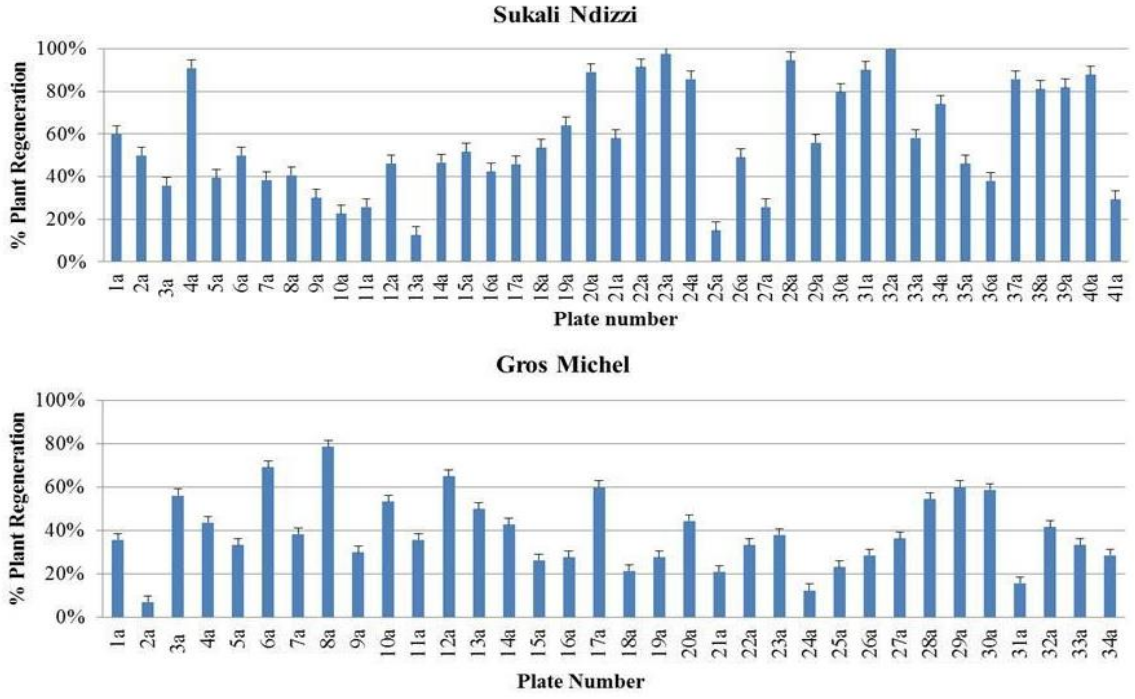

Figure 4. Embryo regeneration efficiency. Graph showing Gros Michel ES-*Pflp* embryo regeneration efficiency and Sukali Ndizzi ES-*Pflp* embryo regeneration efficiency.

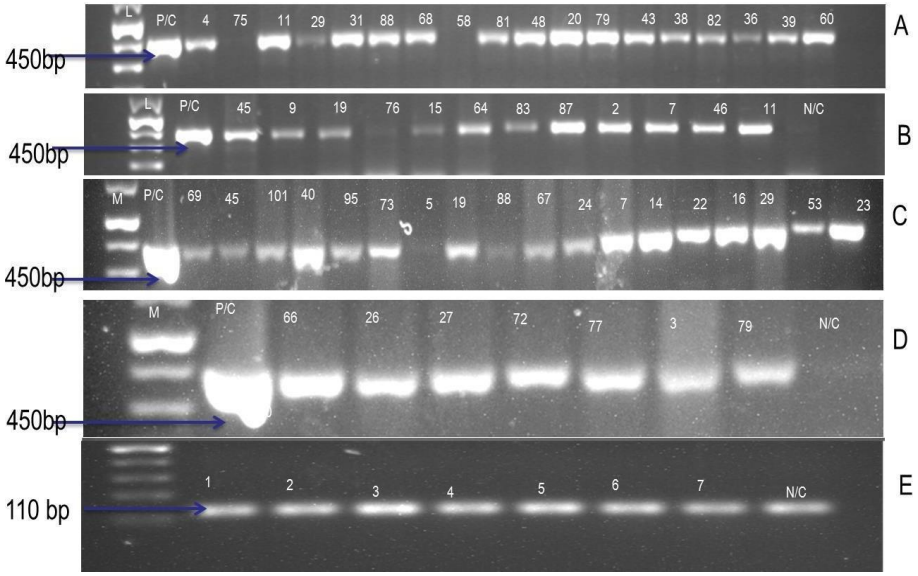

Figure 5. Representative PCR analyses of Sukali Ndizzi and Gros Michel transformed lines. A and B: PCR of Gros Michel Es-*Pflp* lines with *Pflp* specific primers, C and D: PCR of Sukali Ndizzi lines with Pflp primers, E: Sukali Ndizzi ES-*Pflp* and Gros Michel ES-*Pflp* lines with housekeepingprimers (25s).

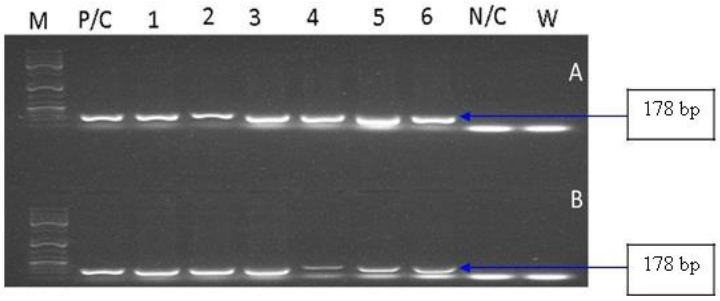

Figure 6. Representative RT-PCR picture of lines transformed with ES-*Pflp.* A: Sukali Ndizzi lines with ES-*Pflp*, B: Gros Michel lines with ES-*Pflp.*

*Growth parameters of Sukali Ndizzi ES-*Pflp *lines*

Table 2 displays growth measurements for various Sukali Ndizzi ES-*Pflp* transgenic banana lines, before they were subjected to disease testing. It appears that, as compared to non-transformed plants, most transgenic lines (line 19, 65, 80, 43.47, 9, 101.88.87, 31, 15.62, and 82) had a lower average number of leaves. In addition, when compared to the control plants, the Sukali Ndizzi ES-*Pflp* transgenic lines 64, 7 and 81 showed significantly greater mean leaf numbers. However, the analysis of variance (ANOVA) revealed that there were no statistically significant changes (p=0.27708) in the number of leaves between the transgenic and non-transformed plants in terms of leaf number between the two groups.

In terms of leaf length, the bulk of the Sukali Ndizzi ES-*Pflp* transgenic lines had significantly longer average leaf lengths than the non-transformed plants, when compared to the control plants (Table 3). Although the observed differences in leaf length between transgenic and non-transgenic plants were statistically significant (p=0.274748), the results of the ANOVA revealed that they were not statistically significant (p=0.274748). Comparing the leaf diameter of Sukali Ndizzi ES-*Pflp* lines to the control line showed superiority, with no statistically significant differences (p=0.190951).

As indicated by ANOVA values of p (p= 0.287434), the pseudostem width of Sukali Ndizzi ES-*Pflp* transgenic lines examined varied with no statistically significant difference. The most significant values were found in lines 43, 47, and 7. It was found that there was no statistically significant difference between the Sukali Ndizzi ES-*Pflp* transgenic lines evaluated and the control, with plant height values ranging from 45±1.2cm to as high as 64.670±0.633cm for the transgenic lines screened (p=0.297481).

Growth parameters of Gros Michel Pflp *lines*

Compared to the control line, there was no significant variation in leaf number, leaf length, leaf diameter, or pseudostem width. There was a difference in plant height between the lines and the control line, but the post ANOVA test revealed that the difference was not significant.

Screening of transgenic lines for resistance to BXW disease

It was decided to inoculate the potted plants of Gros Michel and Sukali Ndizzi with Xcm and observe for the development of disease symptoms 60 days after the inoculation. The development of necrosis at the site of inoculation in control plants occurred roughly one week after inoculation in the experimental plants. The first symptoms of illness were detected in the control group 10 days after the vaccination. As illustrated in Figure 11, the transgenic line had a delayed onset of illness symptoms, with some exhibiting no symptoms at all.

Resistance of different Sukali Ndizzi ES-Pflp *transgenic banana lines toBXW disease*

For the Sukali Ndizzi ES-*Pflp* transgenic lines tested, BXW disease resistance was found to be comparable to that of non-transgenic plants when grown in an unstressed environment. Eight days after inoculation in the control line and twenty-four days after inoculation in line 82, which eventually succumbed to the disease after the screening period, the onset of symptoms occurred. Three lines were sensitive to BXW sickness, even though the symptoms of line number 47 and number 82 began later than those of line number 65. Six transgenic lines showed complete resistance to *Xanthomonas campestris* pv. *Musacearum*, namely 24, 101, 87, 2, 31 and 81. In the study, these were the lines that displayed no signs of sickness. Line screenings yielded p=0.034 and α=0.05., a statistically significant difference in resistance between the two lines.

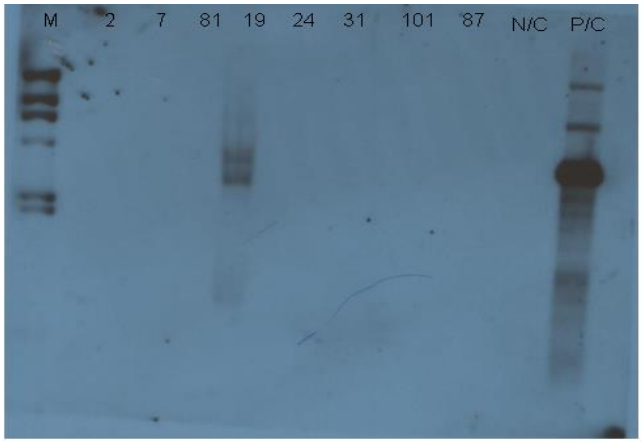

Figure 7. Southern blot analysis of selected lines. M is the molecular weight marker. 2, 7, 81, 19, 24, 31, 101, 87, are the Sukali Ndizzi transgenic lines transformed with ES-*Pflp* gene; NC is the non-transformed control; PC is the plasmid (pBI121 *Pflp*).

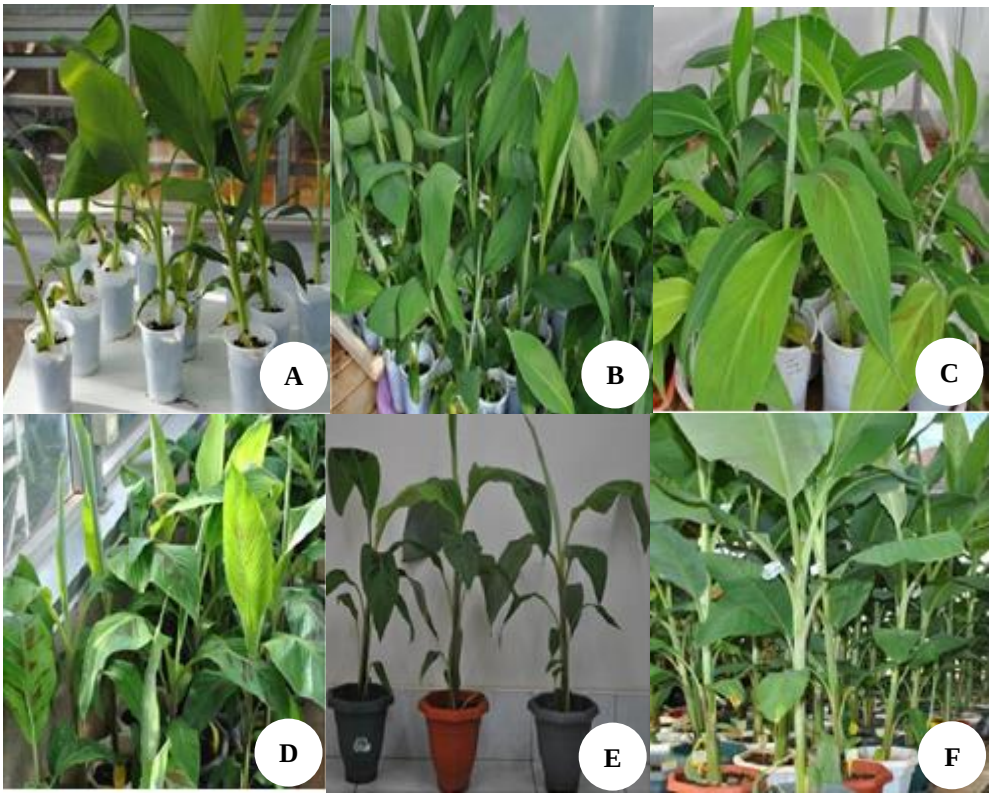

Figure 8. Hardening and potting of Sukali Ndizzi and Gros Michel lines. A and B: One month old Sukali Ndizzi lines in small pots under weaning chamber, C: One month old Gros Michel lines in small pots under weaning chamber, D and E: Three-month-old Gros Michel lines in bigger pots, F: Three-month-old Sukali Ndizzi lines in bigger pots.

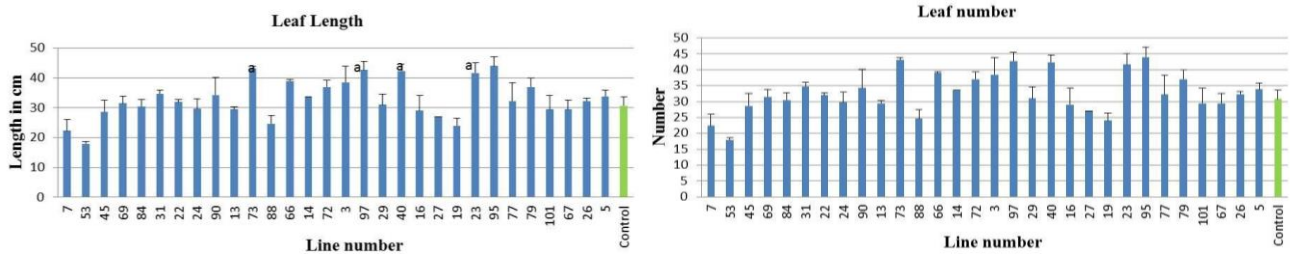

Figure 9. Agronomic traits of Gros Michel ES-*Pflp* in comparison with the controlline. The leaf length of line numbers 73, 97, 40 and 23 showed significant variation with the control line; p=0.00 where α=0.05. There was no significant variation in leaf number of the lines measured in comparison withcontrol line; p=0.054334 where α=0.05.

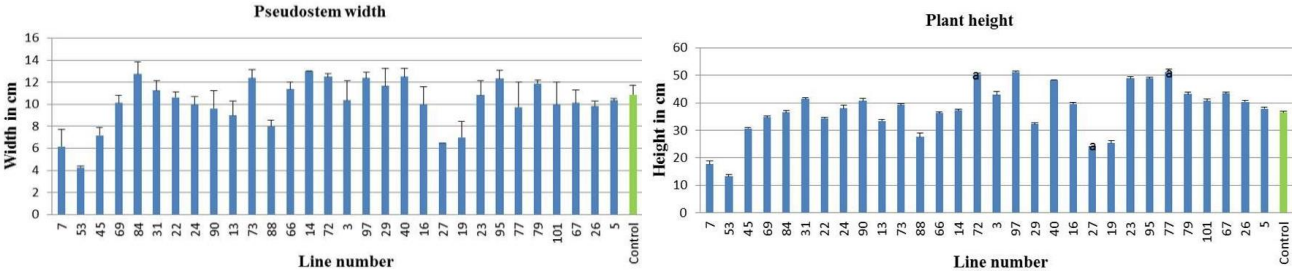

Figure 10. Agronomic traits of Gros Michel ES-*Pflp* in comparison with the controlline.

Figure 11. Representative picture of the screening process. A and B: Onset of disease symptom showing necrosis at the point of inoculation in Sukali Ndizzi transgenic lines, C: Gross Michel under BXW screening in the glasshouse, D: Plant with inoculated leaf with symptom, E: Plant with one leaf without symptom, F: Completely wilted plant.

Table 2. Growth parameters for different Sukali Ndizzi ES-*Pflp* transgenic and non-transformed banana plants grown underunstressed conditions.

Line no.	19	24	65	80	64	43	47	9	101	88	87	2	31	15	7	62	82	81	Control
No. of leaves	8.75±0.478a	9.0±0.0a	8.67±0.33a	7.67±0.67a	9.33±0.33a	8.5±0.5a	8±0a	8±0.82a	8.5±0.5a	8.5±1.5a	8.5±0.5a	9±0a	7.5±0.5a	8.5±0.5a	10±0a	8.33±0.67a	8±0a	10±0a	9±0.41a
Leaf length	35±3.63a	39.25±2.32a	39.67±3.33a	33.67±5.81a	43.67±0.33a	40.5±1.5a	40±3.9a	22.75±1.44a	31±1a	33±0.9a	40.5±2.5a	41±2a	28.5±0.65a	39±1a	42±0a	38±2a	35±0.8a	42.5±0.5a	33.75±0.63a
Leaf diameter	14.5±1.32	15±1	15.67±0.88	13.67±2.7	18±1.15	15.5±0.5	16±2	9.5±3.5	11.5±3.5	13±3	16±0	17±1	11.5±3.5	15.5±0.5	18±0	15.75±1.03	14±2	18±0	14.5±0.5
PS width	6.25±0.144	7.625±0.125	7±0.5	6±1	7.33±0.33	8±1	8±0	5.5±1.5	6.5±1.5	5.5±1.5	7.25±0.75	6.75±0.3	6±1	7±0	8±0	6.5±0.29	6.5±1	7.25±0.25	7.25±0.353
Plant height	45.38±1.8	62.75±4.48	61.67±4.48	52.67±0.56	60.67±0.67	61.5±2.5	66±3	45±2.2	45±1.2	46±1.1	61.5±0.5	62±3	51±1.3	63.5±0.5	63±0	64.670.6	57±0	61±1	57.5±0.417

Table 3. Growth parameters for different Gros Michel *Pflp* transgenic and non-transformed banana plants grown underunstressed conditions.

Line no.	1	4	5	7	9	12	13	21	29	32	35	36	Control
No. of leaves	7±0	7±0.40	6.67±0.33	6±0.41	6.75±0.25	7.25±0.25	6.25±0.25	5.75±0.63	7.25±0.48	6.75±0.25	6.33±0.67	7.25±0.75	7.33
Leaf length	30.5±2.5	27.5±2.02	31.33 ±0.41	27.75±0.85	30.25±2.39	33±1.08	26.75±2.02	33±2.12	31±1.91	33.25±1.701	23.33±0.67	30.25±2.39	29.67±1.45
Leaf diameter	12.75±1.03	11.25±0.85	12.67 ±1.33	9.75±1.25	10.5±0.5	11.75±1.31	11.5±1.19	14.25 ±0.75	12±1.08	12.5±0.29	11.33±0.88	10.5±1.19	10.33±1.2
PS width	7.63±0.24	5.63±0.38	6±0.5	5.88±0.13	6.25±0.25	7.88±0.66	6.5±0.456	6.75±0.32	6.88±0.13	6.63±0.314	7.17±0.44	6.5±0.5	6.13±0.554
Plant height	33.75±1.18	23.5±1.84	30±0.6	28.75±1.7	29.25±0.29	37.5±3.3	31.75±1.37	33.5±2.9	36.75±1.84	38±1.63	31±1.73	28±1.53	25.75±4.1

Table 4. Assessment of different Sukali Ndizzi ES-*Pflp* transgenic banana lines for superior resistance to *Xanthomonascampestris* pv. *musacearum* under glasshouse conditions.

Transgenic line number	19 α	24 α	65α	80 α	64 α	62α	43α	47α	9 α	101α	88α	87α	2α	31α	15α	82α	81α	7α	Control
Number of days for appearance of the first symptom	10.25±4.11	0.00	9.33±3.2	11±2.1	17.67±1.12	9±2	12.5±1.44	22.5±2.5	14±3.40	0.00	19.5±3	0.00	0.00	0.00	25±3	24.5±2.5	13.5±2.157	33.00	8±2.97
Number of days for complete wilting	0.00	0.00	20±4.3	0.00	13.67±3	13.33±4	21±3.9	36±2.6	28.5±4.4	0.00	30±1.437	0.00	0.00	0.00	23±0.67	47±1	0.00	0.00	11.75±2.35
Disease severity	4.00	0.00	5.00	3.00	2.00	1.00	2.00	5.00	2.00	0.00	3.00	0.00	0.00	0.00	3.00	5.00	1.00	3.00	5.00
Average resistance (%)	0.00	100.00	66.67±1.4	88.89±1.11	59.259±3.03	66.67±2.1	50±2	0.00	50±1.2	100.00	50±1.3	100.00	100.00	100.00	37.5±2.91	0.00	100.00	70.00	0.00
Rating	PR	R	S	PR	PR	PR	PR	S	PR	R	PR	R	R	R	PR	S	R	PR	S

Note: PR: Partial resistance, R: Resistance, S: Susceptible, α: Resistant lines

Table 5. Assessment of different Gros Michel *Pflp* transgenic banana lines for superior resistance to *Xanthomonas campestris* pv. *musacearum* under glasshouse conditions.

Transgenic lines	Mean number ofdays for appearance of the first symptom	Mean number of days for complete wilting	Average resistance (%)	Degree of wilting	Rating
1	5.5±2.25	11±1.1	69.097±2.3	3	PR
4	11.5±2.22	20.5±4.88	50±2.23	4	PR
5	17.33±0.866	20±2	38.095±3.12	4	PR
7	9±3.43	15±1.5	70.83±2.39	3	PR
9	12±3.48	18±1.052	50±2.88	4	PR
12	12±2.27	22.5±1.315	50±2.88	4	PR
13	19±3.34	32.33±1.06	33.33±2.1	4	PR
21	18.5±3.43	32.5±1.19	25±2.5	4	PR
29	4.75±0.475	9.75±0.975	75±2.5	2	PR
32	23.5±1.012	0	72.32±1.95	3	PR
35	14.33±1.43	0	96.67±3.33	1	PR
36	20.75±4.043	35.25±1.263	20±2	4	PR
Control	5.5±0.55	11.75±1.175	0	5	S

Note: PR: Partial resistance; R: Resistance; S: Susceptible

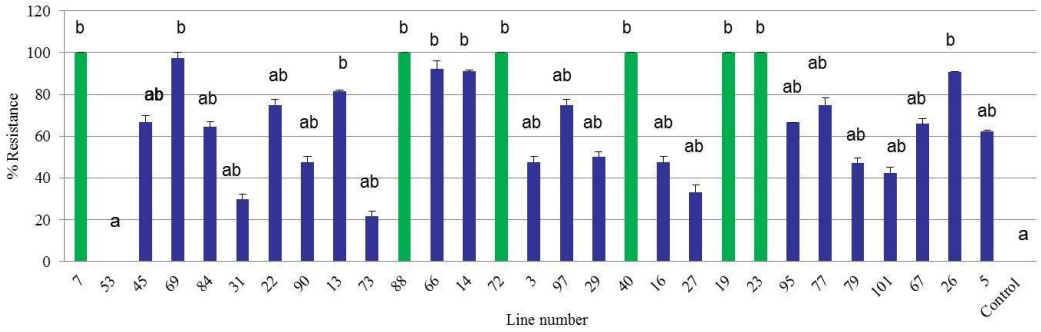

Figure 12. Screening results of Gros Michel ES-*Pflp* lines against BXW disease.

*Assessment of different Gros Michel ES-*Pflp *transgenic banana lines forsuperior resistance to* Xanthomonas campestris *pv.* musacearum *under glasshouse conditions*

A variety of Gros Michel ES-*Pflp* strains were tested for their ability to resist the BXW pathogen. The resistance of each screening line to Xcm is depicted in Figure 12. BXW could infect both the transgenic line 53 and the control line. Numbers 7 through 73 completely resisted the attack of BXW. There was a significant statistical difference in the resistance between the lines screened; p=0.009334 where α=0.05, post-Duncan multiple range tests grouped the lines as indicated above, where any groups that are not significantly different from one another will have the same letter in the grouping column.

Gros Michel Pflp *transgenic banana lines evaluated for superiorresistance to* Xanthomonas campestris pv. musacearum *under glasshouseconditions*

Table 5 compares the beginning of disease symptoms to total wilting and resistance of the different lines examined. For the control line and line number 1, symptoms appeared at 5 days post-inoculation; for line number 32, symptoms appeared at 23 days. None of the BXW-resistant lines tested were utterly resistant. According to the p-value of 0.0335708 for the screening lines, there was a significant difference in resistance among them.

Distribution

With the world's population predicted to quadruple by 2050, there is a pressing need to improve food production to satisfy this growing demand. It is critical to employ methods capable of overcoming cumulative biotic restrictions to maintain food production. These restrictions hamper the ideal genetic production potential for food and fruit crops. Wheat, maize, cassava, and bananas are among of Africa's most important food crops. Despite its immense potential as an export crop, pests and diseases have hampered banana production in recent decades (Jones 2009).

In *Arabidopsis*, extracellular secreted *Pflp* improved harpin-mediated HR disease resistance to *R. solanacearum* and Pectobacterium carotovorum (Lin et al. 2010). In this research, *Pflp* outside the chloroplast had a greater disease resistance. In addition, sea anemone secretion protein is employed in the ES-*Pflp* gene (Lin et al. 2010).

Tissue culture and *Agrobacterium*-mediated transformation were recognized to be difficult for bananas, like any other monocotyledonous plant. In recent years, strategies for transforming banana somatic embryogenic callus and apical meristems using *Agrobacterium*-mediated approaches or particle bombardment have been devised (May et al. 1995; Becker et al. 2000; Ganapathi et al. 2001; Khanna et al. 2004).

Agrobacterium-mediated approaches are chosen because of their ease of use, low cost, capacity to transfer long DNA strands without rearrangement, and low copy number (Huang et al. 2004).

This study employed ECSs from the cultivars Sukali Ndizzi and Gros Michel to create over 100 transgenic lines for each cultivar. Sukali Ndizzi had a greater transformation efficiency (441) than Gros Michel (97.5), indicating that the latter was easier to transform than the former in the two experiments. The embryo regeneration effectiveness of Sukali Ndizzi was higher than that of Gros Michel in both experiments conducted in this study. According to these findings, Sukali Ndizzi is quickly changed and regenerates more embryos than Gros Michel.

Individual regenerated transgenic lines were clonally reproduced, and a few lines were randomly chosen for PCR analysis. Around 97 % of lines carried the ES-*Pflp* gene, indicating that the selection procedure was effective, and few escapes were recovered. The transgene expression patterns were investigated using RT-PCR, which found the transcripts in all transgenic lines tested with no expression in the control line. This demonstrated that the transgene was expressed in the transgenic lines examined, making them appropriate for screening for BXW disease.

Compared to the control line, Southern blot analysis of transgenic lines of Sukali Ndizzi line 19 revealed effective incorporation of two transgene copies. However, other transgenic lines included in this investigation did not demonstrate integration or transgene copy number, which could have been due to technological constraints. Based on these molecular characterization results, Gros Michel and Sukali Ndizzi cultivars were effectively converted using the ES-*Pflp* gene.

The agronomic parameters of the transgenic lines were compared to the control line for each cultivar and revealed no variations in morphology. As a result, transformation with the ES-*Pflp* gene resists BXW disease without altering the plant's physiological state (Tripathi et al. 2014).

Randomly selected transgenic lines of Sukali Ndizzi ES-*Pflp* (19, 54, 65, 80, 64, 62,43, 47, 9, 101, 88, 87, 2, 31, 15, 82, 81 and 7) and Gros Michel ES-*Pflp* (7, 53, 45, 69, 84,31, 22, 24, 90, 13, 73, 88, 66, 14, 72, 3, 29, 40, 16, 27, 19, 23, 95, 77, 79, 101, 67, 26 and 5) were tested for Xcm resistance. Three replicates of 90-day-old potted transgenic lines were intentionally infected with Xcm.

Sukali Ndizzi ES-*Pflp* (18 lines) exhibited variable resistance to BXW, indicating non-targeted gene insertion, a feature of *Agrobacterium*-based transformation. This was validated by statistical analysis, which revealed a significant difference in the resistance to BXW disease between the lines examined. Within 10 days post-inoculation (dpi), non-transgenic control plants displayed symptoms (chlorosis, necrosis, and wilting) and were entirely wilted by 19 dpi. Six of these lines exhibited no symptoms at 60 dpi and were categorized as resistant. Two of the symptomatic transgenic lines wilted, but the remaining 1-3 leaves had symptoms but never entirely wilted, indicating that they possessed some resistance.

Additionally, the Gros Michel ES-*Pflp* screening lines exhibited variable resistance to BXW disease, indicating non-targeted gene insertion, a feature of *Agrobacterium*-based transformation. This was also supported statistically, since substantial differences in BXW disease resistance were observed between the lines evaluated. By 60 dpi, three transgenic lines and three non-transgenic control lines were entirely wilted. Six transgenic lines demonstrated total resistance, while the remaining lines showed partial resistance. Sukali Ndizzi ES-*Pflp* lines demonstrated 33% resistance, while Gros Michel ES-*Pflp* lines demonstrated 20% resistance.

Additionally, twelve Gros Michel transgenic lines (1, 4, 5, 7, 9, 12, 13, 21, 29, 32, 35, 36) transformed with the *Pflp* gene were tested. The non-transgenic lines developed symptoms at 11 days post-inoculation and were fully wilted at 60 days post-inoculation. All screened transgenic lines demonstrated partial resistance, but none showed complete resistance. These results suggest that transgenic Gros Michel banana plants transformed with ES-*Pflp* exhibit increased resistance to BXW than Gros Michel transgenic banana lines transformed with the *Pflp* gene. Previous studies on the ectopic expression of *Pflp* in *Arabidopsis* yielded comparable results (Lin et al. 2010).

In conclusion, the results of this study reveal that the ES-*Pflp* gene can be successfully converted into the Gros Michel and Sukali Ndizzi cultivars. The resistance of the transgenic lines tested against BXW disease varied significantly between the two cultivars, with six lines of each cultivar demonstrating total resistance to the disease. Compared to Gross Michel *Pflp* lines, Gross Michel ES-*Pflp* lines showed more excellent resistance to BXW disease.

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ASIAN J TROP BIOTECHNOL
Volume 18, Number 2, December 2021
Pages: 69-72

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c180203

Short Communication: Comparison of two commercial DNA extraction kit to obtain high quality porcine DNA

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Manuscript received: 25 October 2021. Revision accepted: 17 November 2021.

Abstract. *Wulan DT, Sutanta M, Sophian A. 2021. Short Communication: Comparison of two commercial DNA extraction kit to obtain high quality porcine DNA. Asian J Trop Biotechnol 18: 69-72.* Comparison of two commercial DNA extraction kits, to obtain high-quality porcine DNA, was carried out to give information about the capabilities of the two types of commercial kits used in isolating DNA. This research was conducted to provide information on the quality of DNA isolated from the manufacture of standard DNA from two different types of extraction kits. The kits used in this study were the Mericon Food Kit and the Life River Kit. The method used in this study was the centrifuge column extraction method. The isolated sample was analyzed for purity and concentration using a nano spectrophotometer. The data obtained was then processed statistically to obtain P-value for two mean T-test. Based on the study results, it was found that the average purity and concentration of DNA extracted with the Mericon Food Kit were 2.17 ng/µL and 139.3 ng/µL. Meanwhile, the average purity and concentration of DNA isolated from the Life River Kit were 1.46 ng/µL and 11.9 ng/µL. Based on the statistical analysis of the P-value for the two mean T-test, it was found that the purity and concentration of the two extraction kits were significantly different.

Keywords: DNA, isolation, kit, purity

INTRODUCTION

Currently, medicinal and food products made from animals, like meat, gelatin, fat, and collagen are growing. Along with these developments, new problems have emerged in finding pork content in food and drug products. The content does not match the product claims. In 2020, the Food and Drug Supervisory Agency in the National Agency of Drug and Food Control Indonesia Annual Report stated that from 120 food samples, 7 samples were positive for porcine DNA. This triggered the testing of porcine content in medicinal and food products to ensure that these products are safe and halal for consumption.

Research to develop methods related to DNA detection in food and drug products has been carried out, such as meatball products (Margawati and Ridwan 2010), soft candy (Fadhlurrahman et al. 2015), removing polysaccharides in fruit (Majumder et al. 2011) and capsule shells (Khayyira et al. 2018). A standard or standard porcine genome is needed in every porcine DNA detection test. Porcine DNA isolation from pork has been done previously, using either traditional methods or extraction kits. Suadi et al. (2020) compared the concentrations and purity of the traditional and kit extraction methods. Based on this research, it is known that the concentration of DNA extraction using the traditional method (phenol-chloroform) is higher than the extraction kit method (Mericon Food Kit). Still, the purity obtained from the traditional method is not satisfactory. In addition, the time required for the extraction process with the traditional method is more extended than using an extraction kit (Suadi et al. 2020).

Therefore, this study aims to provide further information on the quality of the extraction results from two different extraction kit methods through the concentration and purity values of the DNA isolates produced. Furthermore, the purity and concentration of both methods were tested statistically using the T-test. Through this T-test, the two methods carried out have significantly different results (Miller and Miller 2010). Therefore, it is hoped that the results of this study can provide additional information regarding DNA extraction techniques in the manufacture of standard porcine DNA using different types of kits.

MATERIALS AND METHODS

Materials

In this study, samples of porcine meat were tested 24 times. In addition, DNA isolation was carried out using the Dneasy Mericon Food Kit (Qiagen) and the Life River Viral DNA/RNA Isolation Kit (Centrifuge Column).

DNA isolation Mericon Food Kit

DNA isolation using the Mericon Food Kit was carried out by weighing 0.2 gr of pork in a 2 mL centrifuge tube and then added 1 mL of Food Lysis Buffer and 25 µL of Proteinase K. Then the tube was incubated in a 60 °C water bath shaker for 20 minutes, then centrifuged at 14000 rpm

for 5 minutes. After that, 500 µL of supernatant was put into a 2 mL tube filled with 500 µL of chloroform, then centrifuged at 14000 rpm for 15 minutes. Then, 350 µL of the top solution was taken into a 2 mL tube filled with 350 µL of Buffer PB, and then the solution was vortexed until mixed. Then the mixture was transferred to a spin column connected to a 2 mL collection tube, and then centrifuged at 14000 rpm for 1 minute. The solution in the collection tube was then discarded, followed by reusing the collection tube to add 500 µL of Buffer AW2. This was then placed in the centrifuge at 14000 rpm for 1 minute. The solution in the collection tube was discarded and the collection tube reused. Furthermore, the drying process was carried out by centrifugation at 14000 rpm for 1 minute. Then, the spin column was transferred into a 1.5 mL tube, with an added 100 µL of Buffer EB, incubated for 1 minute at room temperature, and centrifuged at 14000 rpm for 1 minute (Qiagen 2020).

DNA isolation Life River Kit

DNA isolation using the Life River Kit was carried out by weighing 0.2 g of pork, then put into a 2 mL centrifuge tube. 20 µL of Proteinase K and 500 µL of Lysis Buffer were added. It was then incubated at 56 °C for 10 minutes. After incubation, the sample was added to 500 µL of absolute ethanol and vortexed for 2 minutes. The solution in the binding column was transferred in the collection tube. Next it was then centrifuged at 12000 rpm for 1 minute. The solution collected in the collection tube was then discarded, with the binding column was placed back in the collection tube. This was then washed with Washing Buffer A and centrifuged at 12000 rpm for 1 minute. Followed by being washed with Washing Buffer W and centrifuged at 12000 rpm for 1 minute. It was then washed again with Washing Buffer and centrifuged at 12000 rpm for 3 minutes. The binding column was then transferred to a 1.5 mL tube, with 50 µL of Elution Buffer added, and then let to stand for 5 minutes at room temperature, before being centrifuged at 12000 x g for 3 minutes (Life River 2020).

Purity and concentration analysis

According to the manual, the analysis of purity and concentration was carried out using a DNA Thermo Scientific NanoDrop Spectrophotometer 2000c Spectrophotometer. The first step using nanodrop is to open the nanodrop software, then pipette 2 µL of ER solution, put it on the needle as a blank, and then measure the blank. Then pipette 2 µL of the sample onto the needle nanodrop and measure its purity and concentration.

Data analysis

The data obtained were then analyzed statistically using Microsoft Excel (Office 365) to calculate the P-value for the two mean T-test.

RESULTS AND DISCUSSION

Extraction results with Mericon Food Kit

Using the Mericon Food Kit, porcine DNA extraction was carried out with 12 replications and tested with a DNA Spectrophotometer to produce the purity and concentration values shown in Table 1.

Based on the table of DNA isolation results with the Mericon Food Kit, the sample concentration values were in the range of 133.7 ng/µL-160.1 ng/µL, with an average of 139.3 ng/µL. The value of sample purity measured at wavelength A260/A280 was in the range 2.15-2.18 with an average of 2.17. According to Sambrook et al. (1989), the purity value of DNA isolate above 2.2 indicates that the DNA extraction results still contain RNA contamination. In contrast, if the purity results show a value less than 1.7, it indicates that the DNA extract still contains protein contamination. In this case, the value of DNA purity extracted with the Mericon Food Kit was within that value range. This is also reinforced by the opinion put forward by Abinawanto et al. (2019) and Sophian (2021b), who revealed the same thing where the range of values is the average value of the 4 nucleotides that make up DNA, namely guanine (1.15), adenine (4.50), cytosine (1.51), and thymine (1.47), where if these four nucleotides were read on a nanophotometer they would produce different absorptions when read at wavelengths A260/A280.

Extraction results with Life River Kit

Porcine DNA extraction using the Life River Kit was carried out with 12 replications and tested with a DNA Spectrophotometer to produce the purity and concentration values (Table 1).

Based on Table 2, the results of DNA isolation with the Life River Kit have a sample concentration value in the range of 11.5 ng/µL-12.5 ng/µL, with an average of 11.9 ng/µL, where the concentration value is smaller when compared to the concentration extracted using Mericon Food Kit. This was due to differences in the Lysis Buffer composition from the two types of extraction kits, which resulted in different concentrations of DNA obtained. In this study, the method used followed the manual kit so that the difference in the volume of the solution and the extraction technique is because the kit used is different.

The value of DNA purity isolated from the Life River Kit was in the range of 1.38 ng/µL-1.64 ng/µL, with an average of 1.46 ng/µL. Less than 1.8 ng/µL, which indicates that the results of the DNA isolate still contain protein contamination. The use of RNA extraction/isolation kits in DNA research was carried out by Sophian et al. (2021) on crab processed food products, the results obtained that the average concentration value was 15.46 with a purity value of 2.240. and also, by Sophian (2021a) on processed salted fish products, which obtained the average concentration value at 25.745 with a purity value of 1.729.

In general, DNA isolation/extraction has the same principle between the types of kits used, it's just that the RNA isolation kit uses an RNA carrier as one of the components of the kit used, but this study tries to carry out the extraction kit's performance with different characters. To obtain information about its ability to isolate DNA in the manufacture of porcine standards.

Table 1. DNA isolation result data with Mericon Food Kit.

No. sample	Nanophotometer analysis	
	Purity (A260/A280)	**Concentration (ng/μL)**
1	2.17	160.1
2	2.18	133.7
3	2.18	136.3
4	2.15	139.3
5	2.17	138.4
6	2.18	138.0
7	2.18	137.9
8	2.15	134.8
9	2.18	138.0
10	2.18	138.4
11	2.17	137.2
12	2.17	139.5
Average	2.17	139.3

Table 2. DNA isolation result data with Life River Kit.

No. sample	Nanophotometer analysis	
	Purity (A260/A280)	**Concentration (ng/μL)**
1	1.64	11.5
2	1.49	11.8
3	1.38	12.5
4	1.43	11.9
5	1.47	11.9
6	1.44	11.9
7	1.39	12.0
8	1.44	12.3
9	1.49	12.0
10	1.47	11.6
11	1.43	12.1
12	1.45	11.8
Average	1.46	11.9

Statistical analysis (T-test)

The results of the purity and concentration values from the nanodrop test were then analyzed statistically using the T-test. According to Miller and Miller (2010), in the T-test if the T-count value is greater than the T-table, the two methods compared are significantly different. On the other hand, if the T-count value is less than the T-table, the two methods compared are not very different. The value of the purity of the DNA isolation results with both kits tested t gave the results of t count of 36.6 and t table of 1.8. From these results, the purity values of the two methods differ significantly. For the concentration value of the nanodrop results of the two kits after the T-test, the T-count data is 63.6 and the T-table is 1.8. Based on the results of the T-test, it is known that if T-count is greater than the T-table, this indicates that the two methods are significantly different. The final elution volume difference is a step adjusted to the method used so that this analysis does not use the same steps and volume for each addition of the solution used. This difference will then be the initial information for more comprehensive optimization of further research.

Discussion

In this study, 2 types of extraction kits were used. In the Life River Kit, there was a slight modification in its use by adding proteinase K to the lysis buffer used. In general, the DNA extraction process consists of three main processes, namely cell lysis, which includes a series of stages of destroying cell membranes with the help of proteinase K was used for degrading/cutting the protein structure and SDS (Sodium Dodecyl Sulfate) bind to membrane cell to denature the macromolecule structure. Then, purification is done by giving phenol and CIAA (Chloroform Isoamyl Alcohol), which binds other macromolecules besides DNA, such as carbohydrates, fats, and proteins. And the last step is precipitation, which is a process carried out to separate DNA and water from other organic materials and draw water is done using alcohol, where the separation occurs when centrifugation is carried out (Kado and Liu 1981).

In the Life River Kit, the extraction process is carried out with a slight modification, namely by adding proteinase K at the time of lysis. This enzyme has an essential role in cell lysis. Proteinase K enzyme has another name, endopeptidase K or protease K is a serine protease with a broad spectrum that is commonly used in DNA extraction. Due to the nature of this enzyme that can digest keratin, this enzyme is often referred to as proteinase K. Mini enzyme was discovered in 1974 isolated from the extract of the fungus Engyodontium (Betzel et al. 1993; Mueller et al. 2004). The isolated DNA was analyzed for purity and concentration using a nanophotometer by measuring the absorbance value at wavelength A260/A280. Analysis at the A260/A280 wavelength is a method that is often used to detect DNA (Matlock 2015; Eppendorf 2016; Koetsier and Cantor 2019).

There are 5 nucleotide compositions that makeup DNA or RNA. If the absorbance at wavelength A260/A280 is read, it will show varying values, namely: guanine (1.15), adenine (4.50), cytosine (1.51), uracil (4.00) and thymine (1.47). So, the result of the analysis (1.8-2.0) is the average value of the components that make up the DNA. Meanwhile, to perform RNA analysis, the range value will be greater than this value because in RNA, one of the constituent components is uracil which when compared with DNA composed by thymine, then uracil has a higher value, namely (4.00), so that if averaged, the purity value will be higher when compared with Leninger's DNA (1975).

Based on the research data, it is known that the average purity of the results of the isolation with the Mericon Food Kit and the Life River Kit is 2.17 ng/μL and 1.46 ng/μL, respectively. The concentration of DNA isolated from the Mericon Food Kit was higher (133.7 ng/μL-160.1 ng/μL) than that isolated from the Life River Kit (11.5 ng/μL-12.5 ng/μL). Based on the results of the T-test on purity and concentration, it was obtained data that T-count is more significant than T-table which indicates that the two methods are significantly different.

ACKNOWLEDGEMENTS

The author would like to thank the head of the Center for the Development of Drug and Food Testing for the support given in conducting this research and for the support of laboratory facilities and infrastructure.

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ASIAN J TROP BIOTECHNOL
Volume 18, Number 2, December 2021
Pages: 73-78

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c180204

Activity test on laccase enzyme of fungus *Volvariella volvacea* for decolorization of remazol reactive dye

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Manuscript received: 14 August 2021. Revision accepted: 22 November 2021.

Abstract. *Sari TP, Susilowati A, Setyaningsih R. 2022. Activity test on laccase enzyme of fungus* Volvariella volvacea *for decolorization of remazol reactive dye. Asian J Trop Biotechnol 18: 73-78.* Textile industry waste such as remazol reactive dye is a source of pollution that can damage aquatic ecosystems. This waste is difficult to degrade because it has strong covalent bonds. However, this compound can be degraded biologically (decolorization) using the laccase enzyme oxidoreductase. This study aimed to determine the ability of the laccase enzyme produced by the fungus *Volvariella volvacea* (Bulliard ex Fries) Singer to decolorize remazol reactive dye. The test begins with the decolorization of the dye by the fungal culture of *V. volvacea*, the production and purification of the laccase enzyme with ammonium sulfate, and the decolorization test with the purified laccase enzyme. The results showed that the fungal culture of *V. volvacea* was able to decolorize remazol reactive dye on agar and liquid medium enzymatically. In addition, based on a statistical analysis of ANOVA followed by a *Post-Hoc Test* DMRT method test level of 5%, the results showed that the percentage of decolorization of the three dyes by the laccase enzyme, which had the highest enzyme activity (5.42 U/mL) from the ammonium sulfate purification increased along with increasing incubation time. The highest decolorization percentage occurred in remazol brilliant blue R (RBBR) with an incubation time of 48 hours.

Keywords: Decolorization, laccase enzyme, remazol reactive dye, *Volvariella volvacea*

INTRODUCTION

The textile industry in Indonesia is an industry that continues to grow from year to year. Based on data from Badan Pusat Statistik, in the first quarter of 2019, the growth of the textile and clothing industry was recorded at 18.98%, and this number increased compared to the profit during 2018 of 8.73% (Damayanti 2019). Textile industry waste has polluted the aquatic environment. On the island of Java, water pollution by the textile industry occurs in several watersheds, such as the Citarum River and the Bengawan Solo. Based on data compiled by Greenpeace Indonesia and the Institute of Ecology of Universitas Padjadjaran, it was stated that there was an increase in the concentration of pollutants whose values were higher than usual standards originating from approximately 800 textile factories operating around the Citarum River and the city of Majalaya (Birry and Meutia 2012). Another study around the Bengawan Solo watershed found that the wastewater produced by the textile industry had exceeded the standards of textile industry wastewater quality, namely BOD = 60 mg/L, and COD = 150 mg/L (Lolo and Pambudi 2020).

Most textile industry waste contains synthetic dyes, which are difficult to degrade due to their strong covalent bonds. One of the most frequently used dyes is azo dyes, such as reactive remazol (Kant 2012; Haryono et al. 2018). Azo reactive dyes (N = N) are of significant concern because they are precursors of dyes and form the most significant synthetic dyes with various colors and structures. Therefore, this dye is widely used in the textile industry (Kannan et al., 2013). However, due to the structural properties of aromatic amines, reactive dyes are not readily biodegradable under natural conditions and are not easily removed from waterways by conventional wastewater treatment systems (Babu et al., 2007; Lewis, 2014).

Most of the dye waste cannot be recycled. Still, it can be treated with several handling methods such as coagulation, membrane technology, flocculation, ozonation, Fenton reactive, reverse osmosis, cucurbutyril, electrochemical degradation, and activated carbon. The weakness of these methods lies in the costs involved because it needs many costs for each type of processing. Many textile industries do not treat their waste and throw it directly into waterways. On the other hand, the dye waste can cause turbidity, make the water look bad, smell bad, and prevent light penetration (Widjajanti et al., 2013). Therefore, many kinds of research on waste treatment have been carried out at a reasonably low cost and are easy to do, namely by bio-treatment or processing involving a specific organism (Babu et al., 2007; Sasmaz et al. 2011).

Using organisms, especially microorganisms, in degrading waste is an environmentally friendly alternative. Several microorganisms such as bacteria, fungi, yeast, and microalgae have been found to be able to remove textile dyes (Olukanni et al., 2006; Gowri et al., 2014). These organisms have developed enzyme systems for decolorizing and mineralizing reactive dyes, especially remazol, under certain environmental conditions

(Ekambaran et al., 2002; Faulina et al., 2020). In the case of reactive dye enzymatic remediation, laccase appears to be the most promising enzyme. Laccase has been shown to remove various industrial dyes by decolorizing or decreasing the intensity of the dye (Nyanhongoa et al. 2002).

Laccase enzyme (Lac) is an enzyme that belongs to the ligninolytic enzyme group along with other enzymes, namely manganese peroxidase (MnP) and lignin peroxidase (LnP) (Sharma et al. 2017). Each of these enzymes has the ability to degrade different dyes. Several studies on remazol reactive dyes have shown that high levels of laccase can degrade dyes efficiently (Novotny et al., 2004; Gowri et al., 2014; Sharma et al., 2017). Laccase enzymes are produced mainly by ligninolytic fungi such as *Phanerochaete chrysosporium, Trametes Versicolor, Pleurotusostreatus*, and *Volvariella volvacea* (Novotny et al. 2004; Devi et al. 2012). The fungus *V. volvacea* (Bulliard ex Fries) Singer belongs to the Basidiomycetes group, which usually produces several laccase isoforms. When grown in submerged culture under different conditions, *V. volvacea* produced at least two protein bands with laccase activity. Previous physiological studies have shown that laccase production by *V. volvacea* is induced by copper and various aromatic compounds (Chen et al. 2004a; Ahlawat and Kaur 2018; Akhlawat et al. 2008).

In this study, the activity of the laccase enzyme in the decolorization of remazol reactive dye will be tested using the fungal laccase enzyme *V. volvacea*. This research was carried out in several stages of testing, namely, decolorization of remazol reactive dye with a fungal culture of *V. volvacea* on agar and liquid medium, production and purification of laccase enzyme with ammonium sulfate fraction, measurement of enzyme activity with ABTS substrate, measurement of protein content using the Bradford method, and test decolorization ability of remazol reactive dye with the purified fungal laccase enzyme *V. volvacea*. This research is expected to provide more excellent opportunities in the textile industry to treat waste and reduce pollution.

MATERIALS AND METHODS

The materials used were white-rot fungus *V. volvacea* obtained from the CV. Volva Indonesia (Sleman, Yogyakarta), sterile distilled water, 70% alcohol, 0.5% sodium hypochlorite, potato dextrose agar (PDA), lactophenol cotton blue, $CuSO_4$, streptomycin, dye remazol brilliant blue R (RBBR), remazol black B (RBB), and remazol brilliant red (RBR), glucose, KH_2PO_4, $MgSO_4$, $CaCl_2$, yeast extract, thiamine, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, ammonium sulfate ($(NH_4)_2SO_4$), acetic acid (CH_3COOH) 0.2 M, sodium acetate (CH_3COONa) 0.2 M, $Na_2HPO_4.7H_2O$, $NaH_2PO_4H_2O$, trizma HCl, trizma base, bovine serum albumin (BSA), and Bradford's reagent.

Isolation of the fungus *Volvariella volvacea*

Fungal isolates were made using tissue culture techniques. The fruit bodies of the fungus *V. volvacea* that have been surface-sterilized are prepared. Then, the fruiting body is cut with a sterile knife, and the inside is taken thinly. Thin slices of fruiting bodies were planted in plates containing PDA media and incubated at 30ºC for seven days.

Decolorization test on agar medium

The decolorization ability test of the fungus *V. volvacea* in degrading dyes was carried out by testing its activity in PDA media which had added RBBR, RBB, and RBR dyes with a concentration of 100 ppm and CuSO4 inducer, respectively. Streptomycin was added to the media solution before being poured into Petri dishes (20 µg/1000 mL). 7 days old fungal mycelium agar blocks with a diameter of 0.5 cm were inoculated on the media incubated at 30ºC for 14 days. Colony growth and color fading on the media were observed every day.

Decolorization test on liquid medium

The decolorization test on liquid media was performed by mixing 100 mL of basal media with 100 ppm of dye and $CuSO_4$ as an inducer into a 250 mL bottle. The basal medium used consisted of (gL^{-1}) glucose-5, KH_2PO_4-1, $MgSO_4$-0.5, $CaCl_2$-0.14, Yeast extract-1, Thiamine-0.0025 (Jonathan et al. 2009). The media was inoculated with five pieces of agar mycelium of fungal culture on day 5 (diameter 0.5 cm) and grown under sterile conditions. The cultures were incubated in a shaker incubator at 110 rpm at 30°C (Gbolagade et al. 2006). After 72 hours, the cultures were taken and centrifuged at 5,000 rpm for 1 minute. The absorbance of the supernatant was measured using a UV-Vis spectrophotometer at the maximum wavelength of each dye sample. The result of the absorbance measurement is then calculated as the decolorization value with the following formula: [(A0–At/A0)] x 100, where A0 is the initial absorbance on the first day of inoculation and At is the absorbance at a specific time (Gomaa and Momtaz 2015).

Production of the laccase enzyme of fungus *Volvariella volvacea*

Enzyme production media consisted of 100 mL basal media, 100 ppm dye, 0.05 mM CuSO4, and streptomycin (20 µg/1,000 mL). The medium was inoculated with five pieces of agar mycelium of the fungus *V. volvaceae* and incubated with a shaker at 120 rpm, temperature 30°C, and for as long as nine days. The production medium was separated from the mycelium and centrifuged to obtain a crude extract of the laccase enzyme.

Enzyme purification with ammonium sulfate fractionation

Purification of the laccase enzyme was carried out by precipitation using ammonium sulfate fractionation. The crude extract of the laccase enzyme was precipitated with a certain degree of saturation (0-20%, 20-40%, 40-60%, and 60-80% (w/v)). The crude extract of the laccase enzyme

was added slowly with ammonium sulfate saturation of 0-20% and stirred for 30 minutes. After centrifugation, the enzyme precipitate and supernatant were obtained. The precipitate was dissolved in 100 mM acetate buffer pH 5, as much as 10% of the total volume (Vivekanan et al., 2014; Falah et al., 2018). The supernatant obtained was then precipitated by ammonium sulfate with saturations of 20-40%, 40-60%, and 60-80%. The Bradford method tested the precipitate results and then tested for protein content. Also, the enzyme activity was tested with ABTS as a substrate.

Optimization of temperature and pH for decolorization with purified laccase enzymes

The reaction mixture for decolorization temperature and pH optimization consisted of 500 L of dye with a concentration of 100 mg/L, 300 L of 100 mM acetate buffer pH 4.5, and 100 L of purified enzyme extract. Temperature optimization was carried out at temperature variations of 4°C, 30°C, and 60°C. While the pH optimization was carried out with variations of 100 mM acetate buffer pH 3 and 5, phosphate buffer 100 mM pH 7, and Tris-HCl buffer 100 mM pH 9. Temperature and pH optimization were carried out for 24 hours.

Dye decolorization test with purified enzymes

Decolorization testing using purified enzymes was based on variations in time and dye. The concentration of dye used is 100 ppm. Each reaction mixture was incubated for 3, 6, 24, and 48 hours. Samples were analyzed using a spectrophotometer with a maximum wavelength of each dye used. The maximum wavelengths of RBBR, RBB, and RBR dyes are 595 nm, 590 nm, and 600 nm, respectively (Gowri et al. 2014). After measuring with a spectrophotometer, the percentage value of decolorization was calculated.

RESULTS AND DISCUSSION

Dye decolorization on agar medium

Colony growth was observed to determine the toxic nature of the dye concentration used. In observations within 14 days, inhibition of colony growth only occurred at the beginning of the growth phase. This indicates a process of adaptation of fungi to the media with the addition of dyes. According to Tavcar (2006), the fungus will adapt to the environment in the decolorization process. After the adaptation phase, the enzyme will work by degrading the dye. The results of degradation are nutrients for fungal growth.

The color fading from the decolorization can be seen in the media's color change on the dish's back view. The decolorization process in this test involves the enzymatic mechanism of oxidative enzymes to oxidize the dye to fade. According to Singh and Singh (2010), the decolorization mechanism by fungi can be divided into 2 systems, namely enzymatic and non-enzymatic. The enzymatic mechanism occurs by secreting oxidative ligninolytic enzymes, especially laccase, to mineralize dyes and break down aromatic bonds in dye complexes, resulting in color fading. In contrast, the non-enzymatic mechanism is dye adsorption by the fungal mycelium (adsorption). In this study, the absence of mycelium color change in the fading dye medium indicated that the decolorization process with adsorption did not occur. Therefore, the decolorization process is possible due to the activity of ligninolytic enzymes, especially laccase.

Dye decolorization on liquid medium

The percentage of decolorization is increasing day by day with increasing incubation time (Figures 1-2). On day 15, the percentage of RBBR dye decolorization was 57%. RBB is 49%, and RBR is 45.19%. The dye was easily decolorized by the fungus *V. volvacea*, with the highest decolorization value being RBBR dye.

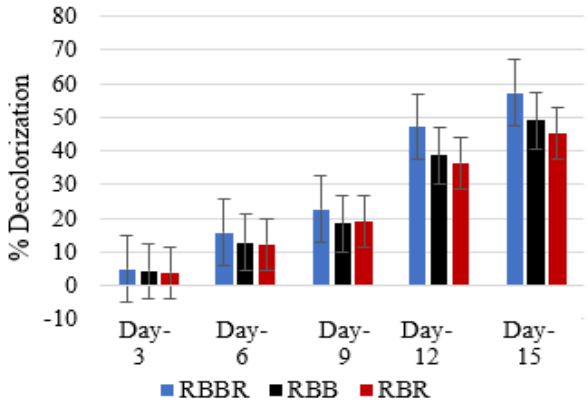

Figure 2. Percentage of decolorization of remazol brilliant blue R (RBBR), remazol black B (RBB), and remazol brilliant Red (RBR) by the fungus *V. volvacea* in liquid culture medium

Figure 1. Color fading by the fungus *V. volvacea* on day 14: A. RBBR, B. RBB, C. RBR

The decolorization process with the fungal culture isolates *V. volvacea* was influenced by the condition of the laccase enzyme, which was still maintained or not denatured by environmental conditions. According to Romero et al. (2006), applying decolorization using fungal culture causes ligninolytic enzymes such as laccase to be protected because they are associated with cells. The decolorization value can be seen from the change in the absorbance value. The change in absorbance value was caused by the dissolution of the dye chromophore group by the activity of oxidative enzymes such as laccase produced by the fungus *V. volvacea*. The laccase enzyme will break down large compounds in the dye into simpler ones with low molecular weights. In addition, the decolorization process is also influenced by the ability of fungal isolates in the adsorption process. According to Martani et al. (2011), fungi can decolorize dyes with non-enzymatic systems through the absorption of dyes by fungal mycelium. The addition of the $CuSO_4$ inducer also influenced the increase in decolorization percentage. According to several studies that have been conducted, $CuSO_4$ is the most commonly used and most effective inducer because the effect of copper produced affects increasing the secretion of the laccase enzyme (Gomaa and Momtaz 2015). The increase in decolorization of remazol dye with liquid culture fermentation was caused by the greater induced laccase enzyme. It was more active in decomposing the substrate, namely the dye (Haedar et al., 2019). According to Erkurt et al. (2007), the decolorization of RBBR dye was influenced by the laccase enzyme, which was induced in the presence of Cu^{2+} compounds in the culture medium.

Enzyme production and purification

The laccase enzyme production resulted in a crude extract with an enzyme activity of 3 U/mL. The production of the laccase enzyme uses a glucose carbon source. The fungus oxidizes glucose to oxalic acid, which accumulates in the medium. The decrease in glucose in the medium was accompanied by a buildup of oxalic acid and a decrease in pH. The extracellular enzymes secreted by fungi are too large to pass through the pores of the fungal cell wall. Calcium in the cell wall is bound by oxalic acid. It can further damage the integrity of the cell wall and provide an opportunity for extracellular enzymes such as laccase to accumulate in the medium (Munir et al., 2001; Bertrand et al., 2013).

To obtain a purer enzyme extract, the enzyme production was precipitated by ammonium sulfate fractionation. The enzyme activity obtained in the 0-20% fractionation was 1.21 U/mL with a total activity of 60.28 U. In the 20-40% fraction, the enzyme activity increased by 3.06 U/mL with a total activity of 137.7 U. For the 40-60% fraction, the protein precipitate was increasing, indicating that the interaction between the enzyme and ammonium salt was getting better. The resulting enzyme activity value was 5.42 U/mL with a total activity of 162.6 U. In the 60-80% fraction; the enzyme activity decreased by 2.17 U/mL with a total of 54.25 U (can be seen in Table 1). This shows that the interaction between the enzyme and the ammonium sulfate salt is reduced.

The protein content of the crude laccase extract to the highest fraction of 60-80% experienced a significant decrease. The initial protein content of the crude extract was 0.114 mg/mL with a total protein of 11.418 mg. The protein content in the purification fraction 0-20% was 0.0649 mg/mL with a total protein of 3.2447 mg. In the 20-40% fraction, the protein content was 0.0271 mg/mL with a total protein of 1.2213 mg. In the 40-60% purification fraction, the protein content was 0.014 mg/mL with a total protein of 0.418 mg. Furthermore, the highest purification fraction was 60-80%, and the protein content was 0.0077 mg/mL with a total protein of 0.1915 mg. The higher the total protein sample, the lower the specific activity. This indicates more protein contaminants than the lactase enzyme, which interferes with substrate conversion to product. Increased protein enzyme purity also occurred in the fractionation with the highest laccase enzyme activity, which was 40-60%. The total enzyme protein in the 40-60% fraction was 0.4182 mg with a specific activity of 112.75 U/mg. In this fraction, the purity of the enzyme increased by 14.8 times with a yield percentage of 54.2% from the purity of the crude extract one time with a yield percentage of 100%. In another study conducted by Chen et al. (2004b), the laccase enzyme from *V. volvacea* isolate had the highest activity value at 80% ammonium sulfate saturation with a total enzyme protein of 7 mg and a specific activity of 3.6 U/mg. In this fraction, the purity of the laccase enzyme is 2.8 times, and the yield is 77% of the purity of the crude extract of the enzyme one time with a yield percentage of 100%.

Table 1. Test data on enzyme activity and protein levels of the fungal laccase enzyme *V. volvacea* purified ammonium sulfate

Fraction	Vol (mL)	Enzyme Activity (U/mL)	Protein (mg/mL)	Total Protein (mg)	Total enzyme activity (U)	Enzyme specific activity (U/mg)	Purity (times)	Yield (%)
Raw Extract	100	3,0000	0,1142	11,4188	300,0	26,2724	1,00	100,00
0-20%	50	1,2056	0,0649	3,2447	60,28	18,5779	0,71	20,09
20-40%	45	3,0600	0,0271	1,2213	137,7	112,7516	4,29	45,90
40-60%	30	5,4200	0,0139	0,4182	162,6	388,8401	14,80	54,20
60-80%	25	2,1700	0,0077	0,1915	54,25	283,2242	10,78	18,08

Table 2. Percentage of remazol reactive dye decolorization by the laccase enzyme of fungus *V. volvacea* in the variation of decolorization time

Dye type	Decolorization percentage on time variation (%)			
	3 hours	6 hours	24 hours	48 hours
RBBR	0,086a±0,075	5,903c±1,361	19,500g±1,290	20,316g±1,007
RBB	2,746ab±1,000	6,483cd±0,991	14,127ef±1,688	15,040f±1,435
RBR	4,450bc±0,667	8,357d±1,189	12,740e±1,846	15,960f±1,576

Dye decolorization with purified enzymes

The optimum temperature for decolorizing remazol reactive dye is 30°C with a percentage of 15.11%. This is aligned with Qin et al.'s (2019) research, which states that the percentage of decolorization by the laccase enzyme of the fungus *Ganoderma lucidum* decreases in conditions below 20°C and above 50°C. In studies with other white-rot fungi, the laccase enzyme's decolorization temperature can be achieved at 40-70°C (Si et al., 2013; Patel et al., 2014; Yan et al., 2014). Based on Murugesan et al. (2009) research, the percentage of decolorization of RBBR dye can be achieved at a maximum temperature of 60°C; beyond that, it can decrease sharply. At the same time, the optimum pH for decolorization is pH 5, with a percentage of 14.86%. According to Si et al. (2013), *Trametes pubescens* laccase enzyme is active and stable at alkaline pH (pH 7.0 to 10.0). However, in another study, most white-rot Basidiomycetes were optimum at a pH between 2.2 to 5.0 (Patel et al., 2014; Yan et al., 2014). This indicates that laccases from different sources have different characteristics. The optimum temperature for decolorizing RBBR dye by fungal laccase *G. lucidum* was optimum at pH 4.0 (Murugesan et al. 2009).

Decolorization percentage data based on a variation of incubation time and remazol reactive dye were analyzed by ANOVA statistical test at a 95% confidence level ($\alpha = 0.05$). If $p < 0.05$ (significantly different), the DMRT further test was conducted at the 5% level. The highest decolorization percentage occurred in RBBR dye, with a decolorization time of 48 hours. Each dye has a different speed of decolorization time. However, the highest overall decolorization percentage value for each dye occurred at 48 hours of incubation (Table 2). Based on interval data from time to time variation, the decolorization percentage indicates the possibility of increasing decolorization after 48 hours. According to Yanto et al. (2019), the percentage of dye decolorization by the laccase enzyme increased with increasing incubation time. The decolorization process will stop after the laccase enzyme has finished reacting to the dyed substrate.

The value of decolorizing remazol reactive dye by purified laccase enzyme is low. In another study, the decolorization of RBBR dye (concentration 100 ppm) with the laccase enzyme from the purified Basidiomycetes isolate ZUL62 resulted in a decolorization value of 62% at a 24-hour incubation time (Falah et al. 2018). In addition, another study using the lactase enzyme from the fungus Trametes Versicolor U97 decolorized RBBR dye by 50% (Sari 2012). This study shows the low percentage of decolorization is caused by possible factors that interfere with the decolorization process. One of them is the low purity level of the laccase enzyme, causing low enzyme activity. In addition, the temperature instability of the purified laccase enzyme causes the enzyme to be easily denatured. According to research, Grassi et al. (2011) stated that the use of purified enzymes was susceptible to unstable conditions compared to the laccase enzyme's raw filtrate. Another possibility that can cause the low value of decolorization is the presence of decomposition products. Based on research by Romero et al. (2006), the product of decomposition in the decolorization process with purified enzymes can cause inhibition of dye degradation.

In conclusion, the laccase enzyme from the culture of fungus *V. volvacea* was able to decolorize remazol reactive dye on agar and liquid culture medium. Decolorization also occurred in the test with the laccase enzyme due to the precipitation of ammonium sulfate with an enzyme activity of 5.42 U/mL. Furthermore, each decolorization value increased with increasing incubation time. This indicates that the laccase enzyme from the fungus *V. volvacea* has the potential as a remazol reactive dye decolorizing agent.

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ASIAN J TROP BIOTECHNOL
Volume 18, Number 2, December 2021
Pages: 79-85

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c180205

Isolation and characterization of lactic acid bacteria from the gut of the grasscutter (*Thryonomys swinderianus*)

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Manuscript received: 14 August 2021. Revision accepted: 22 November 2021.

Abstract. *Agbove MM, Kayang BB, Futse JE. 2021. Isolation and characterization of lactic acid bacteria from the gut of the grasscutter (*Thryonomys swinderianus*). Asian J Trop Biotechnol 18: 79-85.* Intestinal microbiota can affect hosts either beneficially or harmfully. Many efforts have been made to identify and study the microbial community in the gastrointestinal tract of livestock. The grasscutter is a micro-livestock species whose intestinal microflora is yet to be explored. Lactic acid bacteria confer probiotic benefits among the intestinal microflora and are of special interest. This study was conducted to isolate and characterize lactic acid bacteria from the gut of grasscutters in Ghana. Fresh fecal samples were collected from a total of 26 grasscutters comprising nine domesticated grasscutters and 17 wild grasscutters from Ghana. The samples were cultured on MRS agar, and the DNA from 57 bacterial colonies was extracted and sequenced at the 16S rRNA gene to identify the bacteria at the species level using the Basic Local Alignment Search Tool in the National Centre for Biotechnology Information database. Some of the five genera comprising 15 species of lactic acid bacteria (LAB) were identified with ≥99% similarity. Those included *Lactobacillus fermentum* (n = 11), *L. formosensis* (n = 1), *L. salivarius* (n = 11), *L. ingluviei* (n = 9), *L. plantarum* (n= 7), *L. reuteri* (n = 2), *L. taiwanensis* (n = 1), *L. rhamnosus* (n= 1), *Pediococcus pentosaceus* (n = 5), *Enterococcus gallinarum* (n = 2), *E. faecium* (n = 2), *Staphylococcus homini* (n = 2), *Weissella cibaria* (n = 1), *E. hirae* (n = 2), and *W. paramesenteroides* (n = 1). Moreover, all five genera were isolated from the domesticated grasscutters, while only two genera (*Lactobacillus* and *Pediococcus*) were isolated from wild grasscutters. The isolation of *L. ingluviei* is very interesting since this species was originally isolated from birds and is associated with weight gain in mice. The bacteria identified in this study may be important in determining the intestinal health of the grasscutter and should be assessed for their potential as probiotics to improve grasscutter nutrition.

Keywords: Coastal savannah grasscutter, gut, Lactic Acid Bacteria, *Thryonomys swinderianus*

INTRODUCTION

The grasscutter (*Thryonomys swinderianus* Temminck, 1827) is a rodent that is a wild herbivore related to the African brush-tailed porcupine as well as the guinea-pig, chinchilla, and the capybara of South America (Baptist and Mensah 1986). The main habitat is grassland in West Africa, hence the name grasscutter. While in other parts of Africa, it is associated with a cane field called the cane rat (Eben 2004).

Grasscutters occur in Western, Central, and Southern Africa (Mohamed et al. 2011; Adu et al. 2017). In Ghana, the domestication of this rodent started in the 1970s, and it is the preferred game by people living in rural areas in West Africa (National Research Council 1991). In some West African countries, particularly in Ghana, grasscutter meat is a delicacy everyone enjoys, regardless of religion or tribe. Several studies have been conducted on the animal due to its delicacy, popularity, low-fat level, and a high source of protein (Rural Infrastructure and Agro-Industries Division 2012).

Promoting grasscutters as micro-livestock is expected to contribute significantly to reducing malnutrition, which is prevalent in Ghana. Grasscutter rearing depends largely on the peasant farmers who keep these animals in different forms of housing units. Although being the most preferred (Teye et al. 2020) and the most expensive game meat in West Africa (Baptist and Mensah 1986), it has the potential to contribute to both local and export earnings in West African countries. However, dependence on wild grasscutters does not allow for sustainability and planned production for consumption (Yaro et al. 2012), hence the importance of domesticating and rearing them as micro-livestock.

Grasscutters readily adapt to many diets, such as leguminous fodder, roots, food crops, fruits, and grasses (Eben 2004). In the wild, grasscutters derive their nutrients from a variety of feedstuff for the production and maintenance of their body (Wogar 2011). Therefore, the breeder must supply all the needed nutrients in the right proportions in captivity. However, the nutrient requirement of grasscutters is not well known yet. Among the major nutrients, protein is most likely to be deficient in the formulated feed of farm animals because of its diverse roles in the body of animals (McDonald et al. 1996). Furthermore, conventional protein sources, such as fish meals and oil seed cakes (soya bean meal), are very expensive and most farmers cannot afford them.

The unbalanced and low nutritional value of the natural forage and feed fed to the grasscutter usually results in poor performance of the animal. Feeding is essential in animal breeding because it results in good reproduction, health, and growth in the animal (Zougou-Tovignon 2005). Most of the food nutrients are made available to the grasscutter either by microbial or enzymatic activities in the

gastrointestinal tract (Yapi et al. 2012). Despite the grasscutter's good attributes, attempts at its domestication have been marred by malnutrition, under-nutrition, and a high death rate (Yaro et al. 2012).

As a herbivorous animal for potential domestication, sufficient knowledge and understanding of the intestinal micro-flora may provide the much-needed information on how to formulate feed with a suitable probiotic. Those can help increase the animal's feed conversion ratio leading to increased productivity, increased litter size as well as reduced under-nourishment (Adu and Wallace 2004) since the digestion and the absorption of nutrients are dependent on activities of normal microflora of the gastrointestinal tract (GIT) (Draser 1989). Lactic Acid Bacteria (LAB) are prominent among the microflora that promote the health and bioavailability of nutrients in the gut of the grasscutter.

LAB are a group of Gram-positive, anaerobic, non-sporulating, or facultative aerobic cocci or rods, which produce lactic acid as one of their main products of carbohydrate metabolism (Ghoddusi et al. 2011). LAB confers probiotic benefits among the intestinal microflora and is thus of special interest. Markowiak and Śliżewska (2017) defined a probiotic as a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance. LAB forms part of an animal's GIT and boosts the immune system against invading pathogens, availing some nutrients, increasing growth rate, digestion rate, and absorption of nutrients in the intestine, and preventing the onset of some diseases (Florou-Paneri et al. 2013).

Currently, because of regulations restricting antibiotics as feed additives, there is a growing interest in employing probiotics as a suitable alternative in animal production because of issues of generating antibiotic-resistant bacteria. In this regard, LAB offers great promise for use as probiotics. However, it is important first to study the LAB profile in livestock to identify which LAB species to target for use as probiotics for a particular livestock species. Therefore, this study was conducted to isolate and characterize lactic acid bacteria from the gut of the grasscutter in Ghana.

MATERIALS AND METHODS

The sample collection

A sum of 26 fresh fecal samples was collected from both domesticated and wild grasscutters. The domesticated grasscutters (n = 9) were taken from the grasscutter facility at the Department of Animal Science, the University of Ghana, Ghana (n = 4), and Legon Staff village (n = 5). The wild grasscutters (n = 17) were taken from Ghana's Mankessim and Gomoa Abontin regions. Samples were collected using sterile tweezers and dropped into sterile tubes containing De Man, Rogosa, and Sharpe (MRS) broth (Becton, Dickinson and Company, Sparks, USA), then transported as soon as possible to the laboratory. Next, the collected samples were cultured overnight in an incubator at 37°C. The fecal samples were then removed from the MRS broth to get a microbial broth. The resultant MRS broth in the tubes was kept in a plastic bag with an AnaeroPack-Anaero gas generator (Mitsubishi Gas Chemical CO., INC., Japan) and stored at 4°C.

The isolation of LAB

The samples were homogenized and cultured on MRS agar using the streak method. Next, the MRS agar plates were incubated anaerobically using the AnaeroPack-Anaero gas generator at 37°C for 24 hrs. The dominant and discrete colonies were selected (based on morphological characteristics such as color, colony size, roughness or smoothness, and transparency), transferred onto a new MRS agar, and incubated at 37°C for 24 hrs. Next, sub-culturing was further carried out to obtain homogeneous colonies. The homogeneous colonies were then collected into sterilized skimmed milk and stored at -80°C until use.

DNA extraction

A sterilized loop was used for each homogenized colony to collect the colony into 200 µL of a 0.5% triton-X extraction buffer. The resultant solution therein was vortexed and kept at -20°C overnight. Next, the samples were removed, thawed, and placed in a bead beater for about 20 sec at a speed of about 5,000 rpm. Finally, the samples were centrifuged at 13,000 rpm for 5 mins at a temperature of 4°C. A sum of 30 µL of each resulting supernatant was pipetted into a 96 well plate and stored at -20°C until use.

PCR amplification

Amplification of the V3 region of the 16S rRNA gene from LAB was carried out by using primers 27F (5 '- AGA GTT TGA TCC TGG CTC AG 3 ') and 1492R (5 '- GGT TAC CTT GTT ACG ACT 3 ') (Lane 1991). Next, the PCR amplification was performed in a 50 µL reaction mixture containing 0.3 µL of each primer, 2.4 µL of dNTP, 7.5 µL 10×buffer, 3.35 µL distilled water, 0.15 µL Taq DNA polymerase, and 1 µL template DNA. First, PCR amplification was performed on Takara thermal cycler (Takara bio-medical Tokyo, Japan) with denaturing at 94°C for 10 sec, followed by 35 cycles with denaturing at 94°C for 10 sec, extension at 55°C for 30 sec, elongation at 72°C for 30 sec.

PCR product purification and sequence reaction

The PCR product to purify was topped up with distilled water into 100 µL, mixed thoroughly with 500 µL of binding buffer, and then transferred into spin columns. Next, the PCR product was centrifuged at 14,000 rpm for 1 min. Then, 500 µL of washing buffer was added and centrifuged at 14,000 rpm for 1 min. Finally, another 200 µL of washing buffer was added and centrifuged at 14,000 rpm for three mins. Next, spin columns containing the PCR products were transferred into new 1.5 ml tubes, and the flowthrough was discarded. Next, 50 µL of distilled water was added and centrifuged at 14,000 rpm for one minute to elute the purified PCR product. Again, the spin columns were discarded, and the purified PCR product was stored at 4°C.

The sequence reaction was performed in a 15 µL reaction mixture, which contains 3 µL Big Dye, 3 µL of

buffer, 1.5 µL of each primer, and 7.5 µL of purified PCR product. The cycling was carried out in a Takara thermal cycler (Takara bio-medical Tokyo, Japan) with denaturing at 96°C for 10 sec followed by 25 cycles of denaturing at 96°C for 10 sec, extension at 50°C for 5 sec, and elongation at 72°C for 1 min.

Ethanol precipitation and sequencing

The PCR product sequence reaction was purified by ethanol precipitation into a 100 µL reaction mixture, which contained 3 µL of NaOAc (3M), 24.5 µL of distilled water, 62.5 µL of EtOH (99.5%), and 10 µL of the sample. Next, the mixture was vortexed and kept at room temperature for 15 mins and later centrifuged at 3,100 x g for 20 mins. Next, 200 µL of 70% ethanol was added to the plate and mixed by inverting the plate slowly five times to wash the DNA. Next, the plate was centrifuged at 3,100 x g for 5 mins, after which the supernatant was carefully discarded. Next, the plate was wrapped with tissue and cellophane and centrifuged upside down at 800 x g to dry the DNA. After that, 4 µL HIDI formamide was added to each sample to re-suspend the DNA. Next, a sum of 10 µL HIDI formamide was pipetted into a sequencing plate, and 1 µL of each dissolved DNA sample was added. Next, the plate was incubated on a heat block at 95°C for 2 mins and quickly transferred onto a cold block for 5 mins. The samples were finally electrophoresed on an ABI prism 3100 DNA sequencer (Applied Biosystem Division, Foster City, CA, USA).

Sequence analysis

The 16S rRNA sequences method was edited to obtain the required length of about 500-600 bp using Finch TV (www.geospiza.com) and Mega 7 (Kumar et al. 2015) software. Next, the Basic Local Alignment Search Tool (BLAST) was used to align sequences in the GenBank database of the National Centre for Biotechnology Information (NCBI) for species assignment. The strains selected showed ≥99% similarity with 16s rRNA genes in the NCBI GenBank.

RESULTS AND DISCUSSION

Molecular identification of LAB isolates

The sums of fifty-seven isolates were cultured from 26 grasscutter samples and identified as belonging to the following five genera: *Lactobacillus, Weissella, Pediococcus, Enterococcus,* and *Staphylococcus* (Table 1). The domesticated grasscutters, which had 18 isolates of LAB, belonged to the above five genera. In comparison, the wild grasscutter had 39 isolates of LAB, which belonged to just two of the five genera namely *Lactobacillus* and *Pediococcus.* The aligned sequences were identified at the species level (Table 1). Moreover, all the five genera with their species isolated included, *Lactobacillus fermentum* (n = 11), *L. salivarius* (n = 11), *L. ingluviei* (n = 9), *L. plantarum* (n = 7), *Pediococcus pentosaceus* (n = 5), *Enterococcus gallinarum* (n = 2), *E. hirae* (n = 2), *E. faecium* (n = 2), *Staphylococcus homini* (n = 2), *L. reuteri* (n = 2), *L. taiwanensis* (n = 1), *L. formosensis* (n = 1), *L. rhamnosus* (n = 1), *Weissella cibaria* (n = 1) and *W. paramesenteroides* (n = 1).

The cultivable lactic acid bacteria from the gut of the grasscutter

The cultivable LAB from the gut of the grasscutters that were successfully isolated is presented in Table 2, which are 57 LAB isolates belonging to five genera and consisting of 15 species. The species that were found to be common and/or specific to domesticated and wild grasscutters are presented in Table 3. The four LAB species were isolated from both the wild and domesticated grasscutters. These were *L. salivarius, L. reuteri, L. plantarum* and *P. pentosaceus.* And then, four LAB species were also found to belong specifically to the wild grasscutter. These included *L. ingluviei, L. taiwanensis, L. formosensis, and L. fermentum.* However, the seven LAB species were found exclusively in the domesticated grasscutter. These were *L. rhamnosus, E. Hirae, W. cibaria, W. paramesenteroides, S. hominis, E. faecium,* and *E. gallinarum.* There was greater diversity in the domesticated grasscutter than in the wild grasscutter.

Table 4 represents the frequency of LAB isolated from all the grasscutters (domesticated and wild). *L. fermentum* and *L. salivarius* had the highest number of isolates (11 each), representing 19.2% of the total LAB isolates, respectively, with L. ingluviei isolated as the third most dominant isolate representing 15.7% of the total isolates. Next, *L. plantarum* was the fourth most dominant isolate and represented 12.3%. Finally, *L. formosensis, L. taiwanensis, L. rhamnosus, W. cibaria, W. paramesenteroides,* and *Enterococcus hirae* had the least number of isolates (1 each), which represented 1.8% each (Table 4).

The number of LAB isolated from wild and domesticated grasscutter

The domesticated grasscutter had all five genera of LAB that were isolated (Figure 1), while the wild grasscutter had only two genera (Figure 2). However, for the domesticated grasscutter, the five genera represented by 18 isolates included *Lactobacillus* (n = 8), *Enterococcus* (n = 5), *Weissella* (n= 2), *Pediococcus* (n = 1) and *Staphylococcus* (n= 2) (Figure 1) but the wild grasscutter which had two genera and were represented by 39 isolates included *Lactobacillus* (n = 39) and *Pediococcus* (n = 4) (Figure 2).

Discussion

In this study, all 57 isolates (Table 1) belonged to *Lactobacillus, Weissella, Pediococcus, Enterococcus,* and *Staphylococcus* genera. There were 15 individual species isolated as follows: *L. ingluviei, L. fermentum, L. salivarius, L. plantarum, L. formosensis, L. reuteri, L. taiwanensis, L. rhamnosus, W. cibaria, W. paramesenteroides, P. pentosaceus, E. gallinarum, E. hirae, E. faecium* and *S. hominis* (Table 2). Although within the isolated LAB genera, *Lactobacillus* had the highest proportion of bacteria in all the samples, followed by *Enterococcus* and *Pediococcus*. Conversely, *Staphylococcus* and *Wiessella* had the lowest proportion (Table 4).

Lactobacillus represented 75.4% (Table 4) of the overall LAB isolates. This finding follows the findings of Wang et al. (2014) regarding the isolation of LAB from the gastrointestinal tract of a chicken. Eight species represented the *Lactobacillus* genus (43 isolates): *L. ingluviei*, *L. fermentum, L. salivarius, L. plantarum, L. formosensis, L. reuteri, L. taiwanensis*, and *L. rhamnosus*.

Table 1. The basic local alignment search tool result of lactic acid bacteria isolated from the gut of domesticated and wild grasscutters

Source of animal	Sample ID	Colony ID	Sequence length (bp)	BLAST identity %	E-value	Species
Wild	28 TS	1	675	99	0	*Lactobacillus ingluviei*
Domesticated	13 TS	3	698	100	0	*Weissella cibaria*
		5	796	99	0	*Lactobacillus salivarius*
Wild	30 TS	7	680	99	0	*Lactobacillus ingluviei*
		8	641	100	0	*Lactobacillus fermentum*
Wild	31 TS	9	776	100	0	*Lactobacillus salivarius*
		10	588	99	0	*Lactobacillus ingluviei*
Wild	34 TS	12	565	100	0	*Lactobacillus fermentum*
		13	699	100	0	*Lactobacillus fermentum*
Wild	29 TS	15	686	100	0	*Lactobacillus plantarum*
		16	606	100	0	*Pediococcus pentosaceus*
		17	809	99	0	*Lactobacillus plantarum*
Wild	32 TS	18	579	100	0	*Lactobacillus ingluviei*
		19	576	100	0	*Lactobacillus ingluviei*
Wild	33 TS	20	580	100	0	*Lactobacillus fermentum*
		21	671	100	0	*Lactobacillus fermentum*
		22	576	100	0	*Lactobacillus fermentum*
Wild	43 TS	23	696	99	0	*Lactobacillus fermentum*
		24	704	99	0	*Pediococcus pentosaceus*
		25	680	99	0	*Lactobacillus fermentum*
Wild	41 TS	27	695	100	0	*Lactobacillus plantarum*
		28	690	99	0	*Lactobacillus plantarum*
Wild	42 TS	29	677	99	0	*Pediococcus pentosaceus*
Wild	36 TS	30	677	99	0	*Lactobacillus ingluviei*
		32	678	99	0	*Lactobacillus ingluviei*
		33	791	100	0	*Lactobacillus salivarius*
Wild	35 TS	34	671	100	0	*Lactobacillus fermentum*
		35	695	99	0	*Lactobacillus formosensis*
Wild	37 TS	37	786	99	0	*Lactobacillus salivarius*
		38	642	99	0	*Lactobacillus reuteri*
		39	778	99	0	*Lactobacillus salivarius*
Wild	38 TS	40	778	100	0	*Lactobacillus salivarius*
		41	666	100	0	*Lactobacillus salivarius*
		42	756	99	0	*Lactobacillus ingluviei*
Wild	39 TS	43	666	99	0	*Lactobacillus fermentum*
		44	753	100	0	*Pediococcus pentosaceus*
		45	752	99	0	*Lactobacillus fermentum*
Wild	44 TS	46	749	100	0	*Lactobacillus salivarius*
		47	657	99	0	*Lactobacillus salivarius*
		48	681	99	0	*Lactobacillus ingluviei*
Domesticated	02 TS	49	741	100	0	*Staphylococcus hominis*
		51	800	100	0	*Weissella paramesenteroides*
Domesticated	03 TS	52	691	100	0	*Staphylococcus hominis*
		53	733	99	0	*Enterococcus gallinarum*
Domesticated	01 TS	56	692	100	0	*Lactobacillus salivarius*
		57	777	100	0	*Lactobacillus reuteri*
Domesticated	07 TS	58	668	99	0	*Lactobacillus salivarius*
		59	605	99	0	*Lactobacillus plantarum*
Wild	40 TS	60	642	99	0	*Lactobacillus taiwanensis*
Domesticated	08 TS	61	670	99	0	*Lactobacillus plantarum*
		63	592	99	0	*Lactobacillus plantarum*
Domesticated	09 TS	64	753	100	0	*Pediococcus pentosaceus*
		65	742	100	0	*Enterococcus faecium*
Domesticated	10 TS	66	711	99	0	*Enterococcus hirae*
Domesticated	04 TS	67	728	99	0	*Enterococcus faecium*
		68	566	100	0	*Enterococcus gallinarum*
		69	669	99	0	*Lactobacillus rhamnosus*

Table 2. The cultivable lactic acid bacteria from the gut of the grasscutter

Genus	Species
Lactobacillus	*L. fermentum*
	L. salivarius
	L. ingluviei
	L. plantarum
	L. formosensis
	L. reuteri
	L. taiwanensis
	L. rhamnosus
Weissella	*W. cibaria*
	W. paramesenteroides
Pediococcus	*P. pentosaceus*
Enterococcus	*E. gallinarum*
	E. hirae
	E. faecium
Staphylococcus	*S. hominis*

Table 3. Species of lactic acid bacteria common and/or specific to domesticated and wild grasscutter

Species of LAB	Origin*	
	Domesticated	Wild
Lactobacillus salivarius	√	√
Lactobacillus reuteri	√	√
Lactobacillus plantarum	√	√
Pediococcus pentosaceus	√	√
Lactobacillus ingluviei	x	√
Lactobacillus taiwanensis	x	√
Lactobacillus formosensis	x	√
Lactobacillus fermentum	x	√
Lactobacillus rhamnosus	√	x
Weissella cibaria	√	x
Weissella paramesenteroides	√	x
Staphylococcus hominis	√	x
Enterococcus faecium	√	x
Enterococcus gallinarum	√	x
Enterococcus hirae	√	x

Note: *: √ = isolated, x = not isolated

Table 4. Frequency of lactic acid bacteria species isolated

Genus	Species of LAB	Frequency	Percentage (%)
Lactobacillus	*L. fermentum*	11	19.2
	L. salivarius	11	19.2
	L. ingluviei	9	15.7
	L. plantarum	7	12.3
	L. reuteri	2	3.5
	L. formosensis	1	1.8
	L. taiwanensis	1	1.8
	L. rhamnosus	1	1.8
Weissella	*W. cibaria*	1	1.8
	W. paramesenteroides	1	1.8
Pediococcus	*P. pentosaceus*	5	8.8
Enterococcus	*E. Faecium*	2	3.5
	E. gallinarum	2	3.5
	E. hirae	1	1.8
Staphylococcus	*S. hominis*	2	3.5

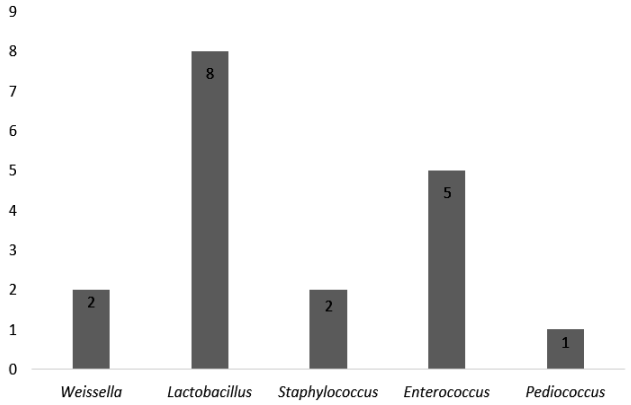

Figure 1. Number of LAB species isolated from domesticated grasscutter

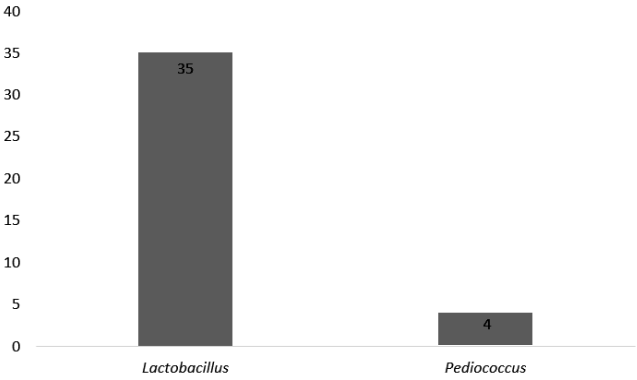

Figure 2. Number of LAB species Isolated from Wild Grasscutter

Lactobacillus fermentum and *L. salivarius* had the highest number of isolates, representing 19.2% of the overall LAB isolates (Table 4), respectively. This result is comparable to the findings of Kobierecka et al. (2017) pertaining to the study of LAB isolated from the chicken intestinal system. *L fermentum* maximized the digestibility of crude protein in piglets (López-Gálvez et al. 2020). *L fermentum* also increased average daily weight, improved intestinal immunity in neonatal pigs (Liu et al. 2014), improved feed conversion and weight gain, reduced diarrhea incidence, and improved meat quality in grower-finishing pigs (Suo et al. 2012). Therefore, it was not surprising to find *L. fermentum* as one of the most dominant lactic acid bacteria in this study since they dominate during the intermediate and final stages of the fermentation (Kogno et al. 2017).

Lactobacillus salivarius was isolated as one of the dominant species (19.2%) of the total LAB isolates (Table 4) as it is fast-growing and a bacteriocin producer. Therefore, from this study, *L. salivarius* was isolated as the dominant species, like the results from the study of LAB being isolated as the dominant species from chicken (Wang et al. 2014) and horse (Rafat et al. 2005). Furthermore, *L salivarius* has been known to develop at a temperature of 37°C. In addition, *L salivarius* has been observed to enhance the breakdown of fiber during fermentation (Yang

et al. 2006) and reduce glucosinolate and crude fiber (Aljoubori et al. 2014).

In the present study, *L. ingluviei* was isolated as the third most dominant LAB representing 15.7% (Table 4) of the total LAB isolates in the wild grasscutter, indicating it is one of the main lactic acid microbiotas of the wild grasscutter. *L ingluviei* may be one of the main lactic acid microbiota in the wild grasscutter. *L ingluviei* has only previously been isolated in birds, specifically in pigeons (Baele 2003) and ostriches (Khan et al. 2007). Quite recently, *L. ingluviei* has been shown to be responsible for weight gain in ducks and chicks (Angelakis and Raoult 2010) as well as in mice (Angelakis et al. 2012). Therefore, *L. ingluviei* would be a lactic acid bacterial species of interest since this is the first isolate from a mammalian species. *L plantarum* represented 12.2% of the total LAB isolates (Table 4).

This species has been widely studied for its numerous application. Wide adaptation capacity with most research focused on its response to the high concentration of lactic acid (Pieterse et al. 2005), low pH and ethanol (De Angelis et al. 2004), bile (Bron et al. 2006), and heat shock (De Angelis et al. 2004).

The least number of *Lactobacillus* species isolated were *L. reuteri*, *L. taiwanensis*, *L. rhamnosus*, and *L. formosensis. L. reuteri* represented 3.5% of the total LAB isolates, while *L. taiwanensis*, *L. rhamnosus*, and *L. formosensis* each represented 1.8% of the total LAB isolates (Table 4).

The genus *Enterococcus* had five isolates represented by three species: *E. Faecium, E. gallinarum*, and *E. hirea*, representing 8.8% of the total LAB isolates (Table 4). This result contrasts with 50% for *E. hirea* isolated from cow feces by Adeniyi et al. (2015) and the 22% reported by Devriese et al. (1987) concerning the isolation of LAB from the intestines of different farm animals. *Enterococcus* species, especially *E. faecalis* and *E. faecium*, have been studied and are known to produce a variety of bacteriocins against many pathogenic bacteria (Ogier and Serror 2008).

The genus *Pediococcus* also had five isolates represented by *P. pentosaceus*. That accounted for 8.8% of the total LAB isolates in this study (Table 4). The *P. pentosaceus* has been shown to reduce fatty liver and obesity (Zhao et al. 2012) in animals. It has also been employed as a food preservative due to its ability to produce antimicrobial agents (Martino et al. 2013).

The genus *Staphylococcus* was represented by *S. hominis*, which accounted for 3.5 % of the total LAB isolates (Table 4). The *S. hominis* is part of the normal microflora of the skin of humans and sometimes animals. Therefore, although it is one of the least isolated species, its presence in the grasscutter may indicate the level of contact between the grasscutters and humans. In addition, the *S. hominis* has been reported to produce bacteriocin, an antibacterial agent against pathogenic *S. aureus* (Sung et al. 2010; da Costa et al. 2016). Therefore, its presence in domesticated grasscutters could have beneficial health implications.

The genus *Weissella* was represented by *W. cibaria* and *W. paramesenteroides*. They constituted 3.5% (Table 4) of the total LAB isolates. Belda et al. (2011) showed that *Weissella* occurred at higher levels in the mid-gut of the European corn borer lab-reared populations than in the field population. *Weissella* species are also commonly found in habitats associated with the human or animal body (Nistal et al. 2012). So it was not surprising to isolate them from domestic but not wild grasscutters.

Although the number of samples collected was not even for both domesticated (n = 9) and the wild (n = 17) grasscutters, the number of LAB genera isolated from the domesticated grasscutter (n = 5) (Figure 1) dwarfed that of the wild grasscutter (n = 2) (Figure 2). The genera *Lactobacillus, Weissella, Enterococcus, Staphylococcus*, and *Pediococcus*, were isolated from the domesticated grasscutter (Figure 1), while only *Lactobacillus* and *Pediococcus* were isolated from the wild grasscutter (Figure 2). The difference in the profile can probably be attributed to the fact that domesticated grasscutters are fed with different feed with different microbial compositions and exposed to human contact, hence the higher number of LAB genera. In contrast, the wild grasscutters select their natural feed, mainly grass, hence the lower number of LAB genera. In addition, the animals' stress conditions (health and diet) were unknown at the time of the sample collection, and these factors usually negatively affect the lactic acid microbiota. Therefore, most species isolated from domesticated grasscutters and wild grasscutters were from the genera *Lactobacillus*.

Although the points of sample collection were also far apart, some LAB species were common to both the domesticated and wild grasscutter, notably *L. salivarius, L. reuteri, L. plantarum*, and *P. pentosaceus*. In addition, four species were specific to domesticated grasscutters, while seven other species were specific to the wild grasscutter (Table 3).

In conclusion, sequence analysis showed that a wide range of LAB species are available for isolation and characterization in the gut of the grasscutter. Furthermore, the domesticated grasscutter had more LAB diversity than the wild grasscutter. The difference in diversity could be attributed to the exposure of domesticated grasscutters to different types of feed and human contact. The isolation of *L. ingluviei* in this study is of great significance since it was only previously isolated from pigeons and ostriches. So this marks the first case of isolation in mammals.

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