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

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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.)

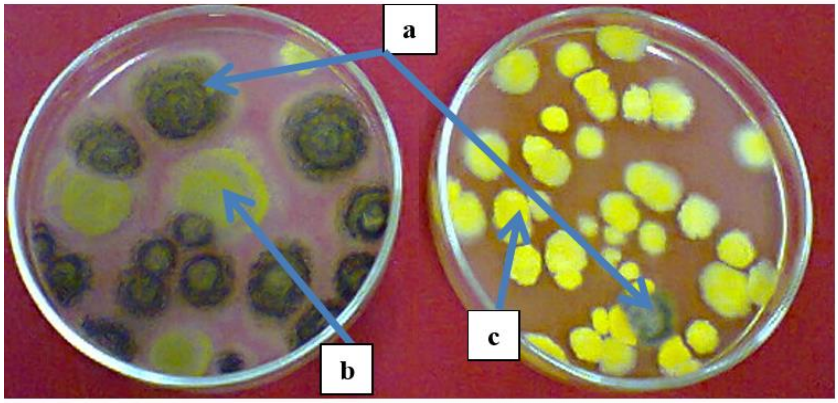

Figure 1. Fungal isolation by dilution method on CYA Media. Left 1:100 dilution, right 1:1000; *Aspergillus flavus* (b), *A. niger* (a), and *Eurotium repens* (c).

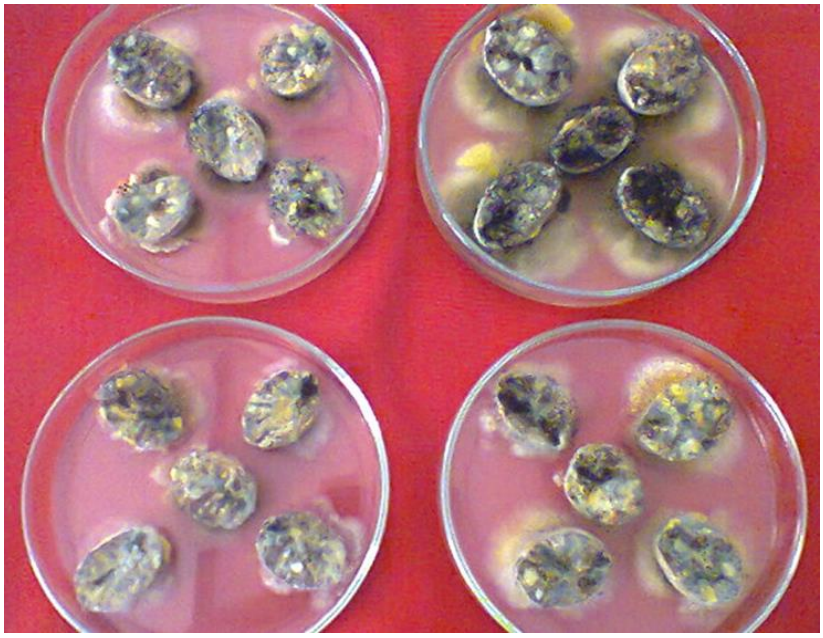

Figure 2. Fungal isolation by the direct plating method on DG18 media.

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*.

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