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DEVELOPMENT OF DETECTION METHOD FOR SEED-BORNE PATHOGENIC FUNGI ON RICE SEED USING FIBER OPTIC FLUORESCENCE SPECTROSCOPY

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ABSTRACT

Aspergillus flavus, *Bipolaris oryzae* and *Fusarium semitectum* are fungi that infect rice crop both in field and storage. Seed health testing is conducted in order to prevent or to minimize risks caused by seed-borne pathogens. One of potential technology which can be developed as detection method is fiber optic fluorescence spectroscopy. The research aimed to develop an alternative detection method more rapid, accurate, and simple for seed-borne fungal pathogen based on secondary metabolites by using fiber optic spectroscopy fluorescence. This research covered: preparation of pure isolates of *A. flavus*, *B. oryzae*, and *F. semitectum*; calibration of fiber optic fluorescence spectroscopy and fungal secondary metabolite analysis by Py-GC-MS; and detection of *A. flavus*, *B. oryzae* and *F. semitectum* on rice seed. The data were analyzed by Spectrasuite Ocean Optic software. Fiber optic fluorescence spectroscopy was able to detect fluorescence emission of fungal metabolites after rice seed were incubated for 24 hours. Each fungus produced specific secondary metabolite when exposed to violet or ultraviolet light which has specific fluorescence emission. *A. flavus* produced aflatoxin B1 at 440 nm, *B. oryzae* produced ophiobolin A at 534 nm, and *F.semitectum* produced beauvericin at 510 nm.

Keywords— *Aspergillus flavus, Bipolaris oryzae, Fusarium semitectum*, secondary metabolite, wavelength

INTRODUCTION

By using high quality seed will increase crop production quantity and quality. However, seed-borne pathogenic fungus is one of the limiting factor in terms of high quality seed availability and escalation of food crops production. Seed-borne pathogens are able to affect seed quality criteria, particularly seed health. Seed-borne pathogenic fungi can interrupt physiological processes in various plant growth phases by the change of nutrition composition and seed biochemistry, also become an inoculum source both in storage and field. Several seed-borne pathogenic fungi can produce mycotoxin within seeds which is hazardous for human and animal health (Mew and Gonzales 2002; Ali 2017).

Detection and identification are essential actions in plant diseases control in order to reduce the risk of loss due to pathogenic fungi. The current method which have been using as standard seed health testing and recommended by International Seed Testing Association (ISTA) is a blotter test (ISTA 2010). According to Duan *et al.,* (2007), blotter test method is simple, accurate, and effective seed health testing. The weaknesses of blotter test method are the time of incubation, high seed germination and rapid saprophyte growth (Mathur *et al.,* 1989). Fluorescence spectroscopy with fiber optic as optic wave guide can be utilized for pathogenic fungi detection and identification which is more rapid, accurate and simple. Fiber optic fluorescence spectroscopy is developed because fungi are known to produce specific metabolite that can produce specific fluorescence emission if being exposed by near ultraviolet (NUV) or ultraviolet (UV) (de Champdore 2017; Hussein 2017). Alcaide-Molina *et al.,* (2009) used laser induced fluorescence detector to detect aflatoxin in standardized growth media and grains. This research aimed to develop an alternative detection method becomes more rapid, accurate, and simple for seed-borne fungal pathogen based on secondary metabolites by using fiber optic spectroscopy fluorescence.

MATERIALS AND METHODS

A. Seed-borne Pathogenic Fungi Isolation and Identification: Four hundred seeds of rice var. Ciherang from Karawang were soaked in NaOCl 1% solution for one minute and were rinsed by aquadest for one minute twice. Afterwards, 25 rice seeds were planted on three filtered paper which had been humidified before by aquadest in petri dish. The petri dishes were put in incubation room beneath NUV lamp radiation with radiation adjustment for 12 hours of light and 12 hours of dark alternately. After being incubated for 24 hours, those petri dishes were transferred into freezer with a temperature of -20 ^{o}C for 24 hours in order to prevent seedling development and make monitoring activities simpler. Furthermore, petri dishes were transferred back into incubation room for 5 days. Monitoring was carried out by using stereo microscope and binocular compound microscope. Fungi which grew on seeds were isol-

ated and purified on Potato Dextrose Agar (PDA) Media. Species identification was conducted based on morphological characteristic which was confirmed at Phytopathology Laboratory, SEAMEO BIOTROP, Bogor.

B. The Making of Fiber Optic Probe

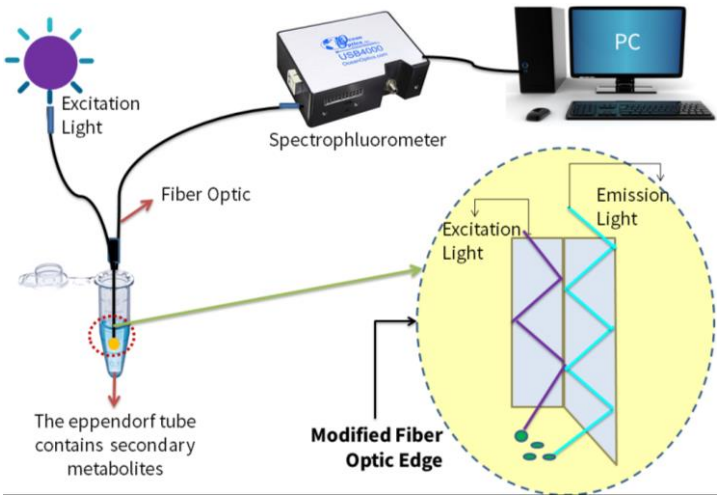


Fig. 1: Pathogenic fungi detection scheme on rice seed by fiber optic fluorescence spectroscopy

This trial utilized 1 m length of two plastic optical fiber, Ocean Optics spectrofluorometer USB 4000 -FL, laser with a wavelength of 405-365nm UV lamp, and computer which has Spectrasuite Ocean Optics software. One of fiber optic edge is equipped with connector, while other edges are not, whereas they are given light source. The censor part is modified by cutting the tip of the fiber optic edge (Figure 1). The using of different light source was based on the maximum of *A. flavus* absorption which lies at a wavelength of 360-370 nm (Yu *et al.*, 2008), meanwhile *B. oryzae* metabolite (Nozoe *et al.*, 1967) and *F. semitectum* (Kouri *et al.*, 2005) are detectable at around a wavelength of 400 nm.

C. Calibration of Fiber Optic Fluorescence Spectroscopy Measurement of Pathogenic Fungal Metabolite Wavelength. Fungal metabolite secondary production was carried out based on Vandermolen *et al.*, (2013) method which was modified. Pathogenic fungal pure isolate was taken as much as two pieces of cork borer and cultured in 100mL erlenmeyer which contained rice extract as Potato Dextrose Broth (PDB) substitution (Jha and Dikshit 2017) and shaken later for 14 days. Fungal suspension was filtered 2 times using 2 sheets of cellulose membrane paper Whatman 0.45 µm followed by Whatman 0.2 µm, then filtered back 2 times using syringe filter 0.45 µm and 0.2 µm. Metabolite result was taken 1 mL and put into an eppendorf tube and excited using a light source of 365nm and 405nm wavelength. Fluorescence emission wavelength of rice extract media and fungal metabolite was measured by spectrofluorometer USB 4000-FL. Some fungal metabolites were analyzed using Py-GC-MS (Pyrolysis Gas Chromatography Mass Spectrometry) at Forest Products R & D Center, Forestry Research and Development Center, Gunung Batu, Bogor.

Fungal Metabolite Fiber Optic Fluorescence Spectroscopy Calibration as Standard. The standard of fungal secondary metabolite fluorescence emission wavelength was obtained with serial dilution from 10^{-1} to 10^{-10} in order to discover the sensitivity of the tools used and the lowest concentration of metabolites which still can be measured by spectrofluorometer. Each fungal metabolite concentration from serial dilution was taken 1 mL and put into eppendorf tube and excited using a light source of 365nm and 405nm. Fungal metabolite fluorescence emission wavelength later was measured and calibrated by Spectrofluorometer USB 4000-FL.

D. Pathogenic Fungi Detection on Rice Seed Production of Fungal Suspension. The seven-day pathogenic fungal isolates which had been grown on PDA media were put into an erlenmeyer flask containing 50mL of aquadest. Suspension was homogenized by magnetic stirrer fore three minutes. The amount of fungal conidia suspension was counted using haemocytometer until reached the density of 10^8 cells/mL.

Fiber Optic Fluorescence Spectroscopy Application on Rice Seed. Rice seeds were sterilized on the surface and fungal inoculation was conducted by soaking rice seeds in target fungal suspension for 24 hours. Seeds were dried manually with the help of wind and put into eppendorf tube containing 1.0mL alternative media. Rice seeds without inoculation were directly put into eppendorf tube containing 1 mL rice extract with 100%,

50% and 25% concentration for 24 hours in order to stimulate rice seed-borne fungal metabolite production. Furthermore, the seeds on media alternative were excited by light source with 365nm and 405nm wavelength to find out emission wavelength from target fungal metabolite.

E. Data Analysis: Data obtained from target fungi detection using fiber optic fluorescence spectroscopy on rice seeds were analyzed using descriptive method by Spectrasuite Ocean software and Microsoft Excel.

RESULT AND DISCUSSION

A. Rice Seed-Borne Pathogenic Fungi: The result of seed health with blotter deep freezing showed that *B. oryzae, F. semitectum* and *A. flavus* are main seed-borne pathogenic fungi with infection rate 8.7%, 8%, 6.8% respectively. *B. oryzae, F. semitectum,* and *A. flavus* are main fungi that infect rice crop both in field and storage (Mew and Gonzales 2002; Manamgoda *et al.,* 2014). Furthermore, *B. oryzae, F.semitectum* and *A. flavus* were utilized as model on rice seed-borne pathogenic fungi detection using fiber optic fluorescence spectroscopy.

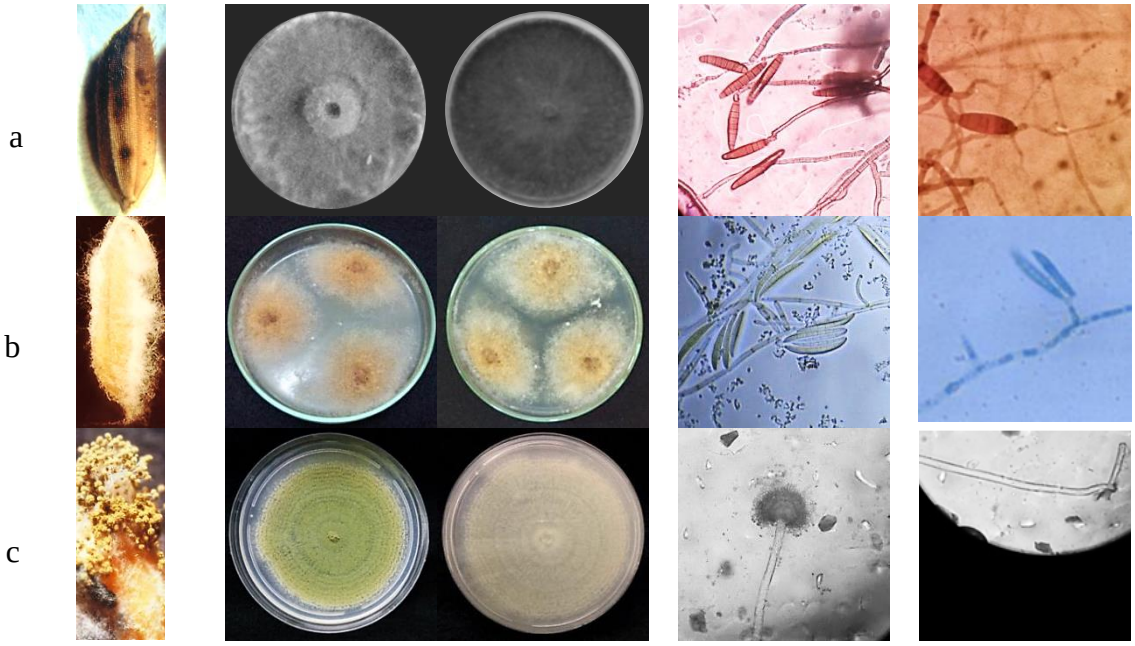


Fig. 2: Macroscopic fungi on seeds, pure isolate appeared in back and front, and micromorphological character of *B. oryzae* (a), *F. semitectum* (b), *A. flavus* (c) magnification of 40 × 10

B. oryzae infection caused brown to black spots symptoms on the surface of rice seed. *B. oryzae* colonies on PDA medium were white and turn into brownish gray as time went by with fluffy and cottony Mycelium. Then, the back side of the PDA media colony were black. Conidiofor grew individually or in small groups, straight, geniculate, and brown. Conidia has 6-14 septats, slightly curved with slightly protruding center and pointed tip, light brown to dark brown, with the size of 40-154.5 × 9.5-24.5 µm. Conidia germinated well at both ends of conidia which showed bipolar characteristics after 12 hours incubation in water agar (Manamgoda *et al.,* 2014) (Figure 2a).

F. semitectum fungus can be found on entire seed surface forming mycelia with white to orange colour and resulted in dark brown infection symptom in seeds. Macromorphological characteristics of PDA media showed solid mycelia growth, white to brownish orange colour as time went by. Fungi produced exudate and soluble pigment with brownish yellow colour. Abundance of macroconidia with straight or slightly curved conidia, generally had 3-5 septate, and slightly long around 23.52-62.10µm. Macroconidia apical shape was tapered or curved while the basal shape was notched. The most distinctive micromorphological character was the abundant production of mesoconidia, having 1-5 septate, straight, spindle-shaped from miselia polyphialides. This mesoconidia was easily observed microscopically in-situ and looked like rabbit ears (Leslie and Summerell 2006) (Figure 2b)

A. flavus colonies on yellowish green-colored PDA media with white mycelium at the edges, forming sporulation roughly coarse rings, and producing yellow exudates and soluble pigments to beige visible on the back of the agar plate. The conidial head had a metula and phialide (biseriate conidial head), the foot cell had more or less perpendicular hyphae and a conidio for terminal into a vesicle. Vesicles had one or two layers and coni-

dia produced by phialides, philiades covering three quarter vesicles. Biserat vesicles had round shape with a diameter of 18-39µm. Conidiofor 450-760 × 9-16µm had coarse texture and colorless texture. The size of the conidia was between 3.5-5µm, globose, slightly coarse, and yellowish green (Klich 2002) (Figure 2c).

B. Analysis of Pathogenic Fungi Metabolite Compound: Pathogenic fungi metabolite compound which had been identified then were analyzed by Py-GC-MS. Py-GC-MS testing result showed that compound with high concentration was an identifier of each fungal metabolite. Aflatoxin B1 was the compound with highest concentration in *A. flavus* metabolite (61%), meanwhile in *B. oryzae* was ophiobolin A (45.80%), and beauvericin compound (16.26%) in *F. Semitectum* metabolite. Aflatoxin B1 compound is a main mycotoxin which is generally found in cereal products and is carcinogenic also hepatotoxic to both humans and animals (Yu *et al.,* 2008). Ophiobolin A is produced specifically by *B. oryzae*. Ophiobolin biological activity showed antibiotic, cytotoxic and carcinogenic traits. Ophiobolin A is the main cause of brown spot disease on rice which can interrupt rice physiological processes since nursery until harvest time (Nozoe *et al.,* 1967). Beauvericin has cytotoxic trait to humans, animals and plants. Insecticidal activity is also shown by beauvericin.

The formation of mycotoxins is affected by physical, chemical and biological factors. Physical factor consists of temperature and humidity. Chemical factor consists of air composition and substrate trait. Biological factor is associated with host species. Nutrients such as carbohydrates as a source of energy, proteins, and minerals are required for the formation of mycotoxins. Aflatoxin production is associated with high carbohydrate concentrations, such as rice and at a lower level in vegetable oils from cotton seed. Rice is an excellent growth medium for the fungus to test the toxigenic potential of pathogenic fungi (Tanaka *et al.,* 2007).

C. Fiber Optic Fluorescence Spectroscopy Calibration: Measurement of metabolite fluorescence emission wavelength which was produced by *A. flavus* was 440nm, *B. oryzae* 534nm and *F. Semitectum* 510nm (Figure 3) on excited rice extract medium by UV light (365 nm) for *A. flavus* and violet (405 nm) for *B. oryzae* and *F. Semitectum*. Fluorescence emission wavelength of rice extract medium at various concentrations which was excited by UV light was 476 nm and with violet light was 502 nm (Figure 4). Each metabolite fluorescence emission wavelength was different with rice extract medium and wavelength of excitation light.

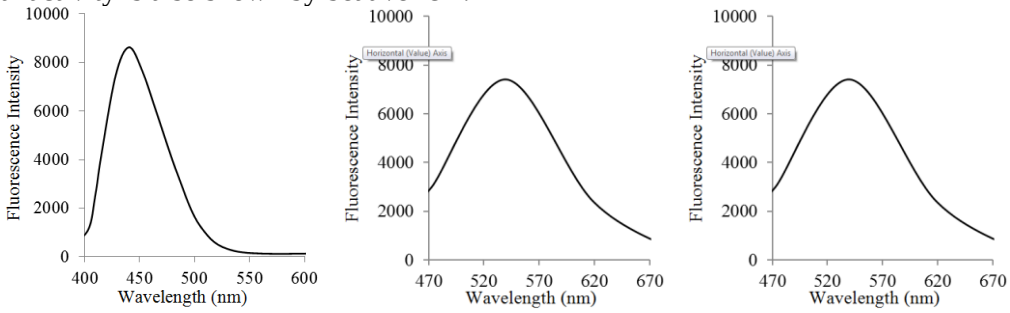


Fig. 3. Metabolite fluorescence emission spectrum *A. flavus* (a), *B. oryzae* (b), and *F. semitectum* (c)

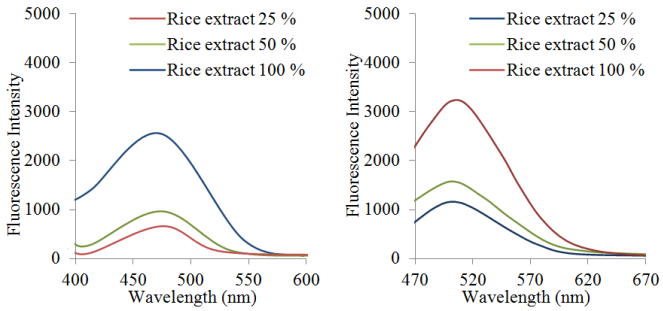


Fig. 4. Fluorescence emission spectrum of rice extract media on 100%, 50% and 25% concentration excited by UV light (365 nm) (a) and violet (405 nm) (b)

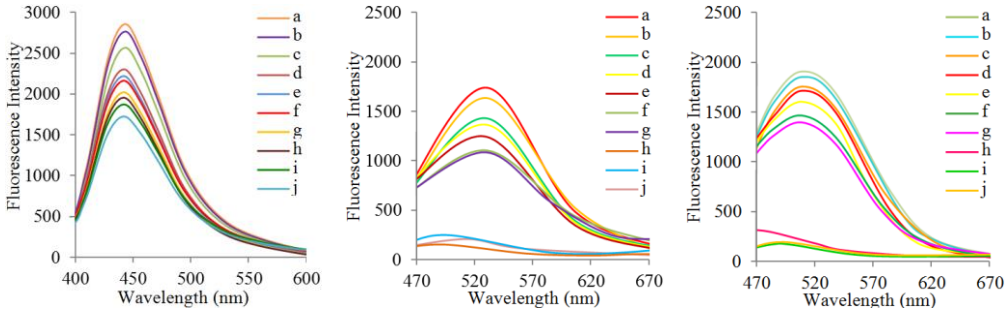


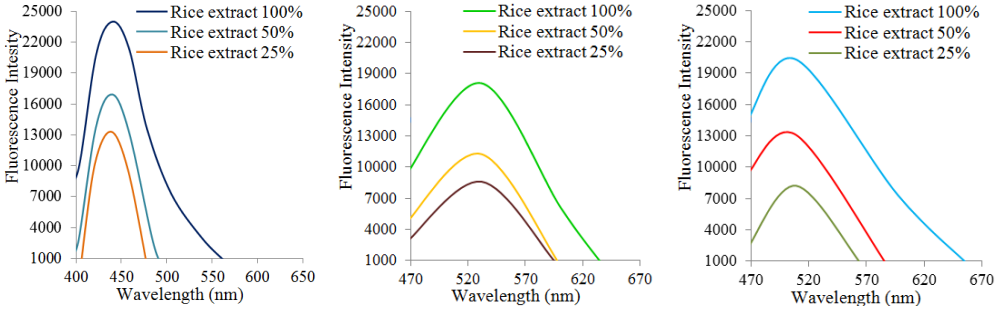
Fig. 5. Metabolite fluorescence emission spectrum *A. flavus* (A), *B. oryzae* (B), and *F. semitectum* (C) result of serial dilution 10^{-1} (a), 10^{-2} (b), 10^{-3} (c), 10^{-4} (d), 10^{-5} (e), 10^{-6} (f), 10^{-7} (g), 10^{-8} (h), 10^{-9} (i), 10^{-10} (j)

The dilution of the fungal metabolites with concentrations of 10^{-1}-10^{-10} was performed to determine the minimum limit of detectable metabolite concentrations. *A. flavus* metabolite emission wavelength at all concentrations showed the same result of 440 nm (Figure 5a). Each of *B. oryzae* and *F. semitectum* metabolites produced varying emission wavelengths (Figure 5b and 5c). It showed that all dilution concentration of *A. flavus* metabolite can be measured by optical fiber spectrofluorometer, whereas not all concentrations of *B. oryzae* and *F. semitectum* metabolites are measurable. It showed that minimum concentration of *B. oryzae* and *F. semitectum* metabolite which can be detected by optical fiber spectrofluorometer is 10^{-7} Dilution affects the fluorescence intensity of fungal metabolite. The lower the metabolite concentration, the lower the fluorescence intensity (Figure 5). This is influenced by amount of metabolite content in solution. The physical condition of a molecule also affects the absorption process of light energy during excitation, so it produces different fluorescence intensity characteristics (Lakowicz 2006). Bass (2000) said that the fluorescence intensity of a molecule is affected by several conditions such as polarity, ions, electrical potential, temperature, pressure, acidity (pH), hydrogen bonding type, viscosity, and de-excitation inhibitors. The fluorescence intensity is a de-excitation emission result. Therefore, the lifetime at the lowest energy level will affect the magnitude of fluorescence intensity. Furthermore, the fluorescence intensity can be reduced by 10-15% if the sample temperature decreases from 30 °C to 20 °C. Therefore, temperature control is needed in order to make the measurement consistent and more precise (Lakowicz 2006).

D. Detection and Identification of Rice Seed-Borne Pathogenic Fungi: The measurement of fluorescence emission wavelength on rice seed was carried out with and without artificial inoculation (Figure 6-9). Each fungus was detected and identified based on wavelength 440 nm (*A. flavus*), 534 nm (*B. oryzae*), and 510 nm (*F. semitectum*) on rice extract media with concentrations of 100%, 50%, and 25% each incubated for 24 hours. Figure 7 showed fluorescence emission of fungal metabolites wavelength on rice seed which were inoculated artificially for 24 hours in each conidia suspension of the fungus. Each detected metabolite was the same and consistent with fluorescence emission wavelength during calibration.

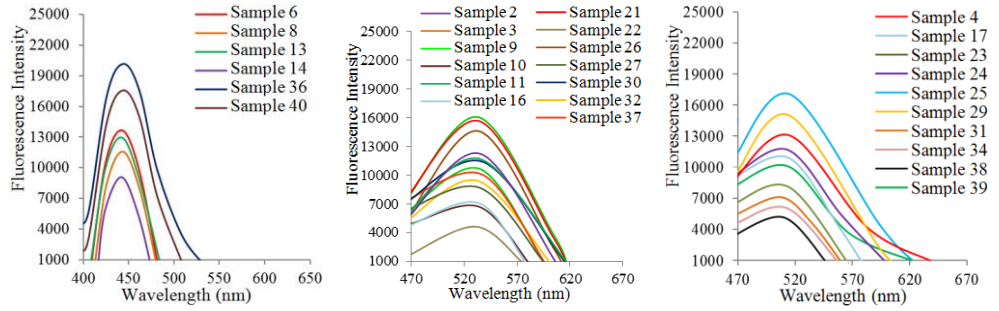
The difference of rice extract media concentration gave effect on the amount of available nutrients to the fungus in producing the metabolites. The lower the concentration of rice extract media, the less pathogenic fungi are detected (Figure 8-10). Rice extract media with concentration 25% was able to detect metabolite in 13 samples, at concentration of 50% was able to detect in 20 samples, and at concentration of 100% was able to detect in 29 samples. The carbon element contained in rice extract media is very important for the fungus because the fungus requires large amounts of carbon from other essential elements (Jha and Dikshit 2007).

Rice seed-borne pathogenic fungi detection with fiber optic fluorescence spectroscopy showed the accurate result with 24 hours of incubation. This method is faster and simpler compared to blotter test method which is the standard method of seed borne pathogenic fungi detection. The incubation time needed on blotter method is 7 days (Mathur and Kongsdal 2003). Fast detection method is needed in terms of seed examination. Based on that, fiber optic fluorescence spectroscopy can be developed as seed borne pathogenic fungi detection method.



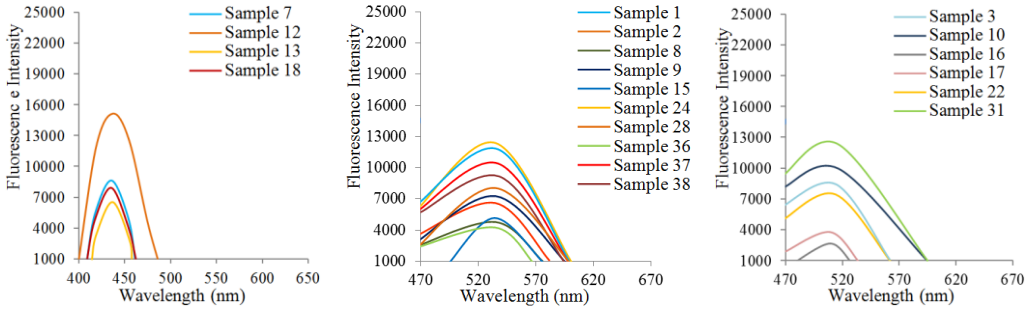
(a) (b (c)

Fig. 6: Fluorescence emission spectrum *A. flavus* (a), *B. oryzae* (b), *F. semitectum* (c) on rice seed which were artificially inoculated in the medium of rice extract concentrations of 100%, 50%, and 25% for 24 hours.



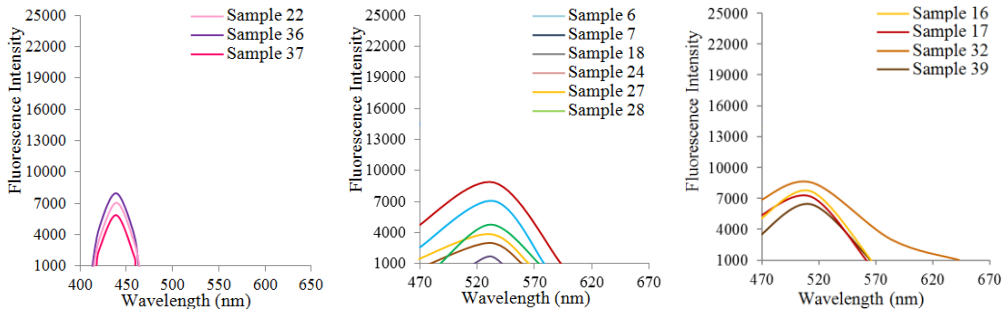
(a) (b (c)

Fig. 7: Fluorescence emission spectrum *A. flavus* (a), *B. oryzae* (b), *F. semitectum* (c) on rice seed which are not artificially inoculated in 100% concentration of rice extract medium incubated for 24 hours



(a) (b) (c)

Fig. 8: Fluorescence emission spectrum *A. flavus* (a), *B. oryzae* (b), *F. semitectum* (c) on rice seed which are not artificially inoculated in 50% concentration of rice extract medium incubated for 24 hours



(a) (b) (c)

Fig. 9: Fluorescence emission spectrum *A. flavus* (a), *B. oryzae* (b), *F. semitectum* (c) on rice seed which are not artificially inoculated in 25% concentration of rice extract medium incubated for 24 hours.

CONCLUSIONS

The main compound and identifier metabolite of *A. flavus* was aflatoxin B1, whereas in *B. oryzae* was ophiobolin A, and beauvericin compound in *F. semitectum*. Based on testing by fiber optic fluorescence spectroscopy method, *A. flavus* fungus was able to be detected on emission wavelength 440 nm, whereas *B. oryzae* on 534 nm, and *F. semitectum* on 510 nm. Fiber optic fluorescence spectroscopy sensitivity depended on metabolite concentration and nutrient of fungi growth media. *B. oryzae* and *F. semitectum* had the lowest metabolite concentration which were 10^{-7}, whereas *A. flavus* was able to be detected until 10^{-7}. Rice extract media which were used as alternative media could stimulate fungal metabolite production at incubation of 24 hours. The lowest media concentration level, the less the fungus was detected.

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REFERENCE

Alcaide-Molina, M., J. Ruiz-Jiménez, J. Mata-Granados, L.M. de Castro, High through put aflatoxin determination in plant material by automated solid phase extraction online coupled to laser induced fluorescence screening and determination by liquid chromatography triple quadrupole mass spectrometry. J. Chromatogr. A. 1216(7): 1115–1125 (2009).

Ali, A.M, Activity of thuja (*Thuja orientalis*) alcoholic extract in inhibition *Aspergillus flavus* growth and detoxification of Aflatoxin B1 in contaminated corn seeds. Pak. J. Biotechnol. 14: 503-506 (2017).

Bass, D., An introduction to fluorescence spectroscopy [Internet]. [Downloaded at 2016 September 21]. Available at http://homepages. wmich.edu/rsung/files/introflour. pdf (2000).

De Champdore, M., P. Bazzicalupo, L. De Napoli, D. Montesarchio, G. Di Fabio, A new *competttive* fluorescence assay for the detection of patulin toxin. Anal. Chem. 79:751-757 (2007).

Duan, C.X., X.M. Wang, A.D. Zhu, F. Xiao, Testing of seedborne fungi in wheat germplasm conserved in the national crop genebank of China. Agric. Scien. China. 6:682-687 (2007).

Hussein, H.A, Detection of tricothecene of *Fusarium solani* isolates by using HPLC in melon plant. Pak. J. Biotechnol. 14: 213-215 (2017).

[ISTA] International Seed Testing Association, International Rules for Seed Testing Edition 2010. Switzerland (CH): ISTA Co (2010).

Jha, S. and S.N. Dikshit, Alternative culture media for fungal growth using different formulation of plant material. Int. J. Pharm. Bio. 8(1):445-425 (2017).

Klich, M.A. Identification of Common *Aspergillus* Species. Utrecht (NL): CBS (2002).

Kouri, K., M.R. Duxhen, R. Lemmens-Gruber, Effects of beauvericin on the metabolic state and ionic homeostasis of ventricular myocytes of the guinea pig. Chem. Res. Toxicol. 18: 1661-1668 (2005).

Lakowicz, J.R., Principle of Fluorescence Spectroscopy. 3rd ed. New York (US): SSBM (2006).

Leslie, J.F. and B.A. Summerell, The *Fusarium* Laboratory Manual. Iowa (US): Blackwell Publishing Inc (2006).

Manamgoda, D.S., A.Y. Rossman, L.A. Castlebury, P.W. Crous, H. Madrid, E. Chukeatirote, and K.D. Hyde, The genus of *Bipolaris*. Myco. Stud. 79: 221-288 (2014).

Mathur, S.B., O. Kongsdal, Common Laboratory Seed Health Testing Methods for Detecting Fungi. Switzerland (CH): ISTA Co (2003).

Mathur, S.B., K. Singh, J.K. Hansen, A Working Manual on Some Seed-Borne Fungal Diseases. Denmark (DK): DGISP Pr (1989).

Mew, T.W. and P. Gonzales, A Handbook of Rice Seedborne Fungi. Los Banos (PH): IRRI Pr (2002).

Nozoe, S., M. Morisaki, K. Tsuda, S. Okuda, Biogenesis of ophiobolins. Tetrahedron. Letters. 35:3365-3368 (1967).

Tanaka, K., Y. Sago, Y. Zheng, H. Nakagawa, M. Kushiro, Mycotoxins in rice. Int. J. food. Microbiol. 119(1-2):59-66 (2007).

Vandermolen, K.M., H.A. Raja, T. El-Elimat, N.H. Oberlies, Evaluation of culture media for the production of secondary metabolites in a natural products screening program. AMB. Express. 3:71 (2013).

Yu, G., R.T. Yu, K.E. Pankin, Fluorescent properties of aflatoxins in organized media based on surfactants, cyclodextrins and calixresorcinarenes. J. Analyt. Chem. 63(8):751-755 (2008).