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# APPLICATION OF FIBER OPTIC FLUORESCENCE SPECTROSCOPY METHOD FOR DETECTION OF *Fusarium verticillioides* and *Penicillium oxalicum* ON MAIZE SEED

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## ABSTRACT

Maize as food crop provides high economic value in Indonesia and other countries. Maize production is still low caused plant diseases. *Fusarium verticillioides* and *Penicillium oxalicum* are two of fungi that infect maize in storage. One of potential technology 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-fungi based on secondary metabolites. This research covered the preparation of pure isolates of the fungal, calibration of fiber optic fluorescence spectroscopy, detection of fungal on maize seed, fungal secondary metabolite analysis by Py-GC-MS, and data analysis by *Spectrasuite Ocean Optics*. Fiber optic fluorescence spectroscopy was able to detect fluorescence emission of a metabolite fungal after maize seeds were incubated for 24 hours. Metabolite of *F. verticillioides* and *P. oxalicum* produces fluorescence at 505 nm and 552 nm.

Keywords— *secondary metabolite, seed-borne disease, seed quality, wavelength*

## INTRODUCTION

Maize (*Zea mays* **L.**) is one of an essential food crop in the world, after wheat and rice. Maize is the main carbohydrate source in several countries. According to Statistics Indonesia data, maize production in 2015 was 20.67 million tons or increased by 1.66 million tons compared to 2014. The escalation of maize production in 2015 increased by 8.72% due to the escalation of the harvested area by 4.18% or an expansion of 160 thousand hectares. Maize production is still low although it tends to increase, it shows that the utilization of high-quality maize seed among farmers has not developed as expected.

One of the external factors which affect the quality of food and feed on maize is a fungal infection *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp. Those fungi are frequently found on maize in the storage (Muis *et al*., 2002). The early infection occurs on the silking phase in filed, then carried by seeds into storage (Schutless *et al*., 2002). Those fungi then evolve and produce mycotoxins. Therefore, feed material becomes damaged and low-grade. The economic loss which is caused by mycotoxins is difficult to calculate precisely. However, *Food and Drug Administration* (FDA) of United States of America has used computerized simulation to calculate the loss in the United States due to aflatoxin, fumonisin, ochratoxin, and deoxynivalenol on the agricultural crops, livestock and its impact on humans amounting to 932 million dollars per year (CAST, 1989).

Seed-borne pathogenic fungi detection is essential in order to reduce the risk of loss which causes by pathogenic fungi. One of potential technology to be developed in detecting seed-borne pathogenic fungus is fiber optic fluorescence spectroscopy.

Fiber optic fluorescence spectroscopy is developed because fungi are known to produce exact metabolite which will produce specific fluorescence emission if being exposed to near ultraviolet or ultraviolet light. Therefore, this research is important to be conducted in order to develop a fast, accurate and simple alternative detection method for maize seed -born pathogenic fungi based on secondary metabolites produced by fiber optic fluorescence spectroscopy detection.

## MATERIALS AND METHODS

**A. Seeds Health Testing:** Two hundred maize seeds were soaked in NaOCl 1% solution for one minute and were rinsed with aquadest for two minutes twice. Afterwards, maize seeds were planted on three filtered paper which had been humidified before by aquadest in Petri dish. The number of seeds was ten for each Petri dish. Those Petri dishes were put in incubation room beneath the light of near ultraviolet (NUV) with radiation adjustment for 12 hours of light and 12 hours of dark alternaately. On the second day of incubation, Petri dishes were transferred into the freezer with a temperature of -20$^{o}$C for 24 hours in order to stop seedling development and make monitoring activities simpler. Furthermore, Petri dishes were transferred back into incubation room for five days. Fungi symptoms and morphology were examined under the microscope. Target fungi which grew then were purified on PDA media in order to get fungi pure isolate (Mathur *et al*., 1989).

**B. Fiber Optic Fluorescence Spectroscopy Calibration**

**B.1. Fungal Metabolite Filtering:** The testing was carried out by following Margino, (2008) method which had been modified. Fungi pure isolate was taken were taken as much as two pieces of cork borer and cultured in 100mL Erlenmeyer which contained maize extract as alternative media for Potato Dextrose Broth (PDB) substitution and shaken later for 14 days. The fungal suspension was filtered by a vacuum pump, Buchner funnel, syringe filter and two sheets of cellulose membrane paper Whatman 0.45µm, furthermore filtered by the same amount of cellulose membrane paper Whatman 0.2µm.

**B.2. Measurement of Seed-borne Pathogenic Fungi Metabolite Emission Wavelength:** The metabolites produced were taken 1 mL and put in Eppendorf. Metabolites then were excited by violet light of 405 nm wavelength. Afterwards, metabolite fluorescence emission wavelength of target fungi was measured by *Spectrofluorometer* USB 4000-FL.

**B.3. Fungal Target Metabolite Fiber Optic Fluorescence Spectroscopy Calibration as Fungal Metabolite Standard:** Fungal metabolite target was diluted with aquadest. Dilution series was carried out in order to obtain metabolite concentration of $10^{-1}$-$10^{-10}$. It was conducted to obtain metabolite fluorescence emission wavelength of target fungi. Afterwards, each of target fungal metabolite from several concentration was taken 1mL and put into Eppendorf. Metabolites on Eppendorf were excited with violet light 405 nm. Metabolites fluorescence emission wavelength of target fungi on several concentration was measured and calibrated by *Spectrofluorimeter* USB 4000-FL.

**C. Seed-borne Fungi Detection on Maize Seeds**

**C.1. Fungal Suspension Creation:** Seven days fungi target isolate which had cultured on PDA were put on Erlenmeyer which contained 50 mL sterile aquadest, then being homogenized with a vortex mixer for 12 hours.

**C.2. Fiber Optic Fluorescence Spectroscopy Application on Maize Seeds:** Maize seeds were soaked in target fungi suspension for 24 hours. Furthermore, maize seeds were put into Eppendorf which contained maize extract media with a concentration of 25%, 50% and 100% for 24 hours and being excited by violet light at 405 nm wavelength to find out light emission of the fungal metabolite. Metabolite fluorescence emission wavelength of target fungi on maize seeds was measured by Spectrofluorometer USB 4000-FL (fig -1).

This disease symptom was marked with the pink to brown of surface maize seeds, sometimes followed by growth of mycelium such as pink cotton. While

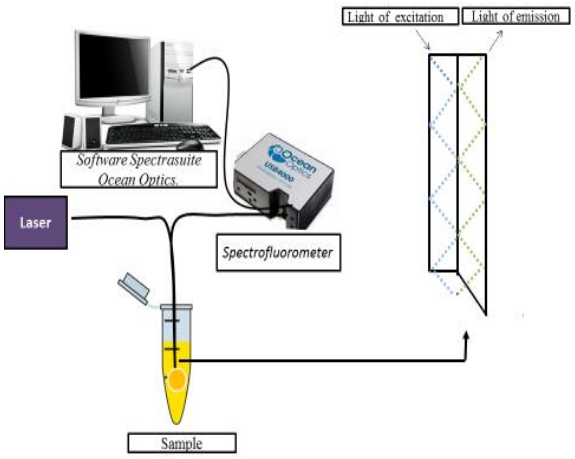



**Fig. 1:** Schematic application of fiber optic fluoresce nce spectroscopy

D. **Fungal Metabolite Compound Analysis:** Fungal metabolite compounds were analyzed qualitatively using Pyrolisis Gas Chromatography-Mass Spectrometry (Py-GCMS) at Institute of Forestry Research and Development, Bogor.

**E. Data Analysis:** The data obtained from target fungi detection using fiber-optic fluorescence spectroscopy were analyzed descriptively using software Spectrasuite Ocean Optics.

## RESULT AND DISCUSSION

**A. Maize Seeds Health Testing:** The testing result of maize seeds health with blotter test showed that there were 11 species maize seed-born fungi. There were yaitu *Fusarium verticillioides* (40%), *Penicillium oxalicum* (24%), *Penicillium* sp. (14%), *Aspergillus niger (4%)*, *Aspergillus flavus (3.5%)*, *Curvularia* sp. (1.5%), *Rhizoctonia* sp. (1.5%), *Rhizopus* sp. (1.5%), *Alternaria* sp. (1.5%), *Cladosporium* sp. (1%), and *Drechslera* sp. (0.5%). Schutless *et al*., (2002) said that fungi which are frequently found to infect maize in Indonesia are *Aspergillus flavus*, *Fusarium verticillioides*, *Aspergillus niger*, *Eurotium rubrum*. Furthermore, *F. verticillioides* and *P. oxalicum* are used as fungi model in fungal detection using fluorescence spectroscopy method.

*F. verticillioides* is a fungus which has a high level of infection rate on maize seeds. Classification based on Barnett & Hunter, (1998) states that *F. verticillioides* belong to the phylum Deuteromycota, class Sordariomycetes, ordo Hypocreales, family Nectriaceae, and species *F. verticillioides* (anamorf stage). Macroscopically the colonies of *F. verticillioides* on the PDA medium had a pink aerial mycelia color on the front and back.

microscopically, there were transparent hyphae (hyaline) and septa (monocytic). There was a slight macroconidia produced, shaped fusiform, slightly

bent, had a pedicellate on the posterior, 3-5 sided, sized (28-40) x (4-5) µm. Microconidia was in round shape, oval, elliptic to cylindrical, usually single-celled and sometimes cell-shaped, sized (4-8) x (2-3) µm. The microconidium was formed in a false head with a short conidogen (conidiofor) cell and in the chain structure (Fig. 2).

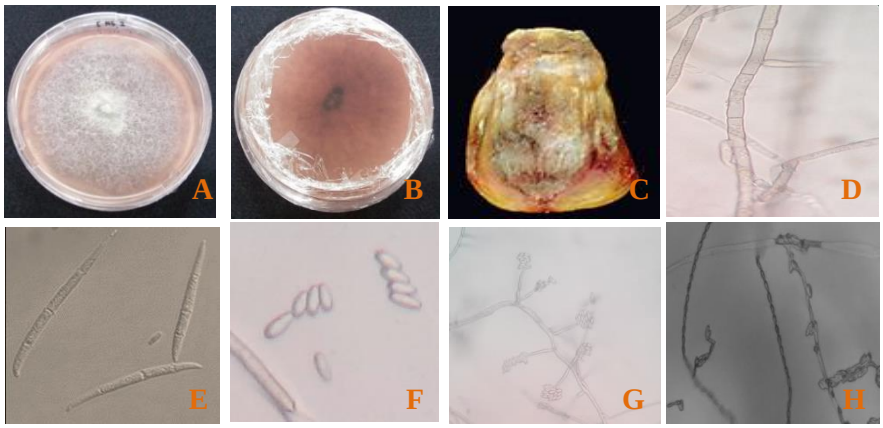



**Fig. 2:** Morphology of front side seven-days *F. verticillioides* on PDA media (A) and back side (B), infection on maize seed (C), hypha (D), macroconidia (E), microconidia (F), microconidia false head (G), microconidia chain (H) 40x magnification.

*P. oxalicum* is a group of Deuteromycota fungi which has the highest infection rate after *F. verticillioides* in maizeseeds. Classification based on Barnett and Hunter (1998) states that *P. Oxalicum* is from phylum Deuteromycota, class Eurotiomycetes, ordo Eurotiales, family Trichocomaceae, and species *P. oxalicum*. Macroscopic characteristics of *P. oxalicum* was an appearance of velvet and dark green colour colonies on PDA and on the other side, the colonies were light yellow. *P. Oxalicum* on maize seeds caused the change of the colour to turquoise and the maize seed became rotten. Microscopically, *P. oxalicum* form resembled penicillate. *P. oxalicum* has transparent hyphae (hyaline), septa (monocytic), and has a width of 4-5 µm.The conidiofor was mostly mononematous and hyaline. The cylindrical-shaped metula carried 3-6 fields. Conidia were formed at the field end and form a basipetal strand. Conidia were round or semi-round (3.0-3.5) x (2.5-3.0) µm, greenish, and smooth-walled (Fig. 3).

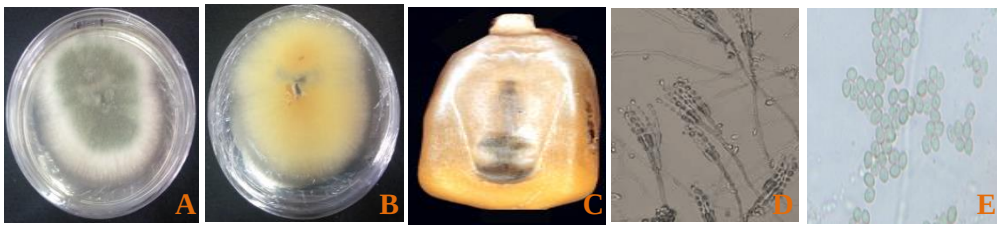
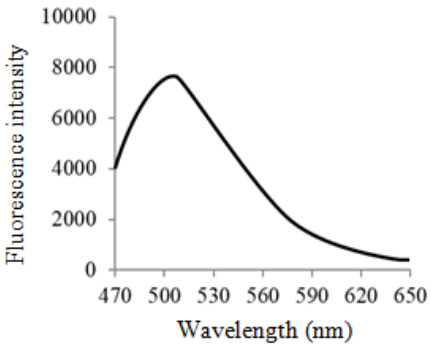



**Fig. 3:** Morphology of front side seven-days *P. oxalicum* on PDA (A) and back side (B), infection on seed maize (C), structure of *P. oxalicum* (D), conidia (E) 40x magnification.

**B. Spectroscopy Fluorescence Calibration:** Excitation of *F. verticillioides* and *P. oxalicum* metabolites with violet light (405 nm) resulted in fluorescence emissions at 505 nm and 552 nm wavelengths as shown in Figure 4. Metabolites of *F. verticillioides* was included in blue-green fluorescence emission spectrum and *P. oxalicum* was Included in green fluorescence emission spectrum Hyedth et al., (2012) reported that metabolite compound in P. oxalicum produce emit green flurorerescence when excited by blue-green flurorescence (500 nm) when excited with ultraviolet light (campolo (2010)



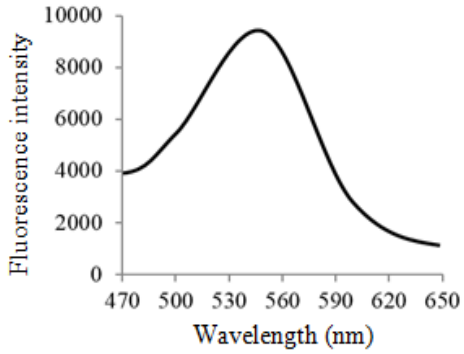



**Fig. 4:** Fluorescence emission spectra of *F. verticillioides* (A) and *P. oxalicum* metabolite (B)

The metabolite emission wavelength measurement result of *F. verticillioides* showed emission wavelengths in the 493-505 nm (Fig. 5A) and *P. oxalicum* showed emission consistent in the 552 nm on several concentrations (Fig. 5B). It showed that not all diluted metabolite concentrations had the same emission wavelength as the *F. verticillioides* metabolite prior to dilution (505 nm). Metabolite emission wavelength dilution result equal to the emission wavelength of *F. verticillioides* was only at dilution $10^{-1}$-$10^{-8}$. *F. verticillioides* metabolite minimum concentration which was able to be detected on fluorescence emission wavelength of 505 nm was concentration $10^{-8}$. Meanwhile, the wavelength of *P. oxalicum* metabolite emissions was able to be measured up to $10^{-10}$ concentration. It shows that spectroflorometer has a high sensitivity level because it can measure the lowest metabolite concentration up to a concentration of $10^{-8}$ on *F. verticillioides* and $10^{-10}$ on *P. oxalicum* dilution series. This is in accordance with Naresh, (2014) assertion that fluorescence testing is sensitive and accurate in each sample. In addition, *F.* vertici llioides and *P. oxalicum* affected the result of fluorescence intensity. The lower the metabolite concentration, the lower fluorescence intensity produced. It was affected by the amount of metabolite content on several physical conditions. Fluorescence intensity on the molecule is affected by several physical conditions such as polarity, ions, electric potential, temperature, pressure, acidity (pH), hydrogen bonding type, viscosity, and quencher. The Fluorescence intensity is related to the concentration of the solution formulated by the equation $I_f = K.\, I_o.C$; $I_f$ is the intensity of fluorescence, K is a constant, Io is radiation intensity of excitation, and C is the concentration of the solution in molar. The intensity of fluorescence is the result of quencher emission so that lifetime at the lowest energy level will affect the magnitude of fluorescence intensity. In addition, the intensity may decrease from 10-15% if the sample temperature decreases from 30°C to 20°C, so it is necessary to adjust the temperature to make the measurement more appropriate (Lakowicz, 2006).

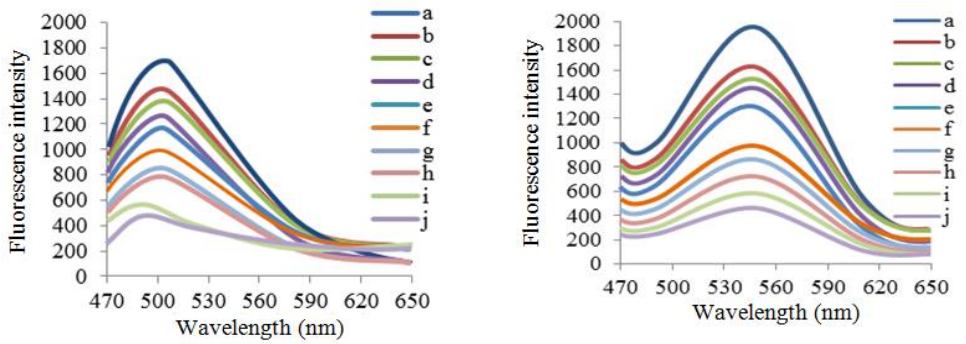



**Fig. 5:** Fluorescence emission spectrum metabolite of *F. verticillioides* (A) and *P. oxalicum* (B), serial dilution result: $10^{-1}$ (a), $10^{-2}$ (b), $10^{-3}$ (c), $10^{-4}$ (d),$10^{-5}$ (e), $10^{-6}$, (f), $10^{-7}$ (g), $10^{-8}$ (h), $10^{-9}$ (i), $10^{-10}$ (j)

Emission wavelength measurement also was conducted on maize extract media as growing media of *F. verticillioides and P. oxalicum*. According to Garraway and Evans, (1984), the nutrients needed by fungi are carbohydrate as an energy resource, protein as a body builder, mineral as substances

that can support the growth of spores. Measurement result showed an emission wavelength of maize extract media was 502 nm as shown in Figure 6. The emission wavelength of not the same with metabolite emission wavelength which produced by the *F. verticillioides* and *P. oxalicum*. It showed that maize extract media had no effect on the measurement of fluorescence emissions produced by the *F. verticillioides* and *P. oxalicum* metabolites.

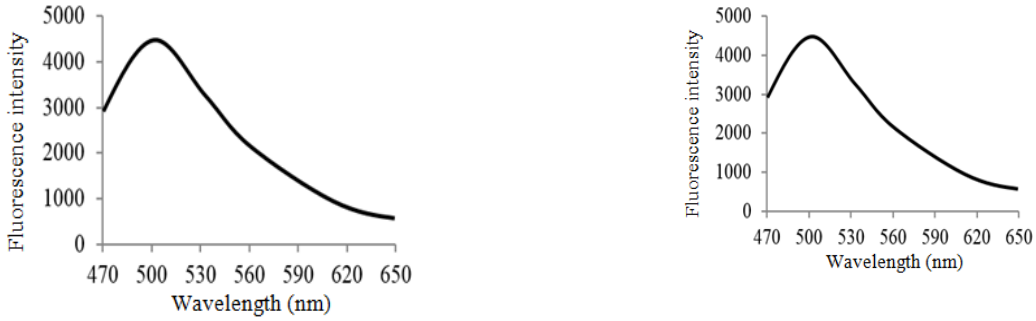
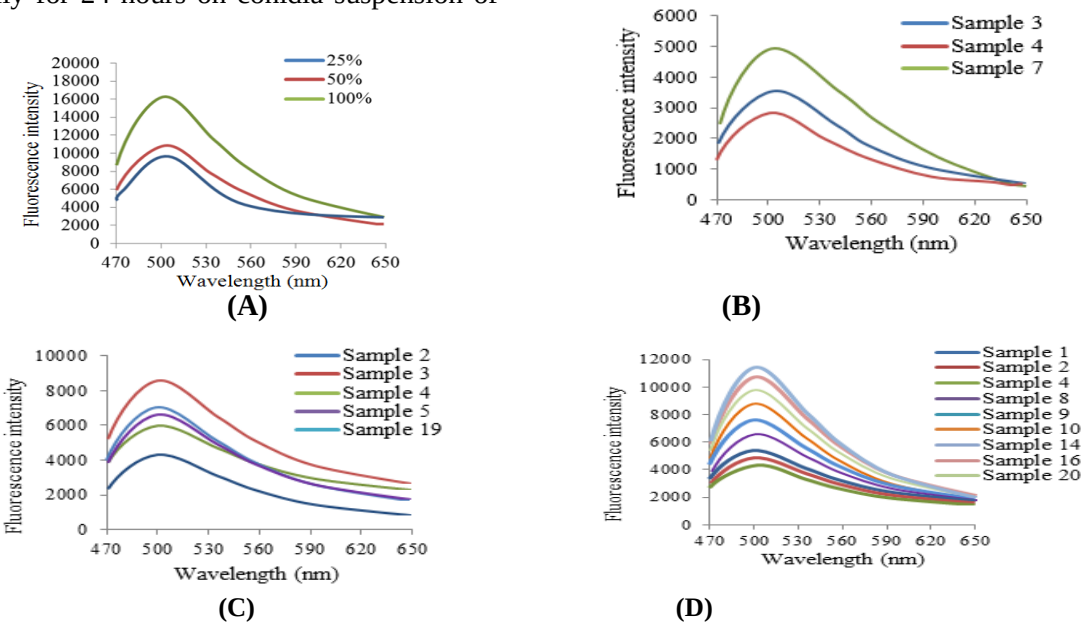



**Fig.6.** Fluorescence of maize extract media

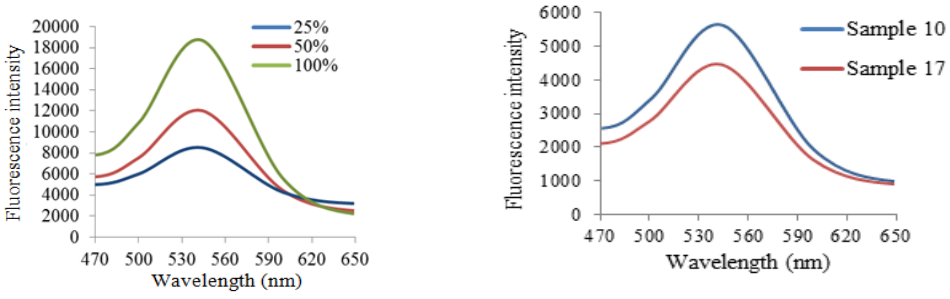
**C. Detection and Identification of *F. Verticillioides* and *P. oxalicum* on Maize Seeds:** Measurement result of fluorescence metabolite emission wavelength produced by *F. verticillioides* and *P. oxalicum* on maize seeds which were inoculated artificially for 24 hours on conidia suspension of *F. verticillioides* and *P. oxalicum* and measurement of emission wavelength on maize seeds without artificial inoculation which were incubated on maize extract media concentration of 25%, 50%, and 100% for 24 hours.



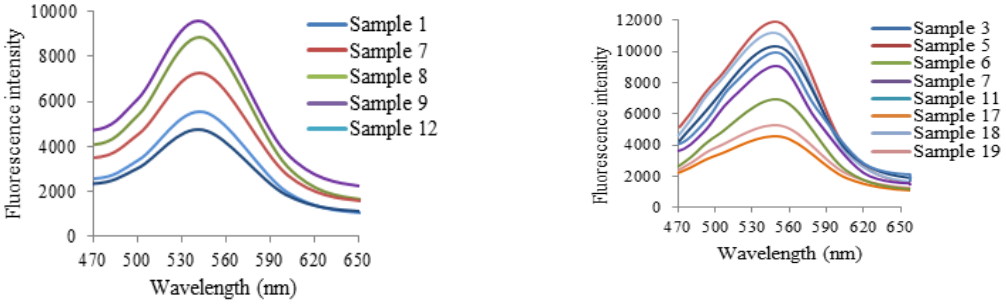

**(A)** **(B)**

**(C)** **(D)**

**Fig. 7:** Fluorescence emission spectrum of maize seed artificially inoculated by *F. verticillioides* (A) and without artificial inoculation soaked in maize extract media concentration of 25% (B), 50% (C), and 100% (D) for 24 hours




**(A)** **(B)**




**(C)** **(D)**

**Fig. 8:** Fluorescence emission spectrum of maize seed artificially inoculated by *P. oxalicum* A) and without artificial inoculation soaked in maize extract media concentration of 25% (B), 50% (C), and 100% (D) for 24 hours

Figure 7 and 8 shows that *F. verticillioides* and *P. oxalicum* metabolites fluorescence emission wavelength on maize seeds with and without artificial inoculation incubated on maize extract media concentration of 25%, 50%, and 100% for 24 hours resulted in emission wavelength of 505 nm and 552 nm. The measurement result of *F. verticillioides* and *P. oxalicum* metabolites wavelength conducted on maize seeds with artificial inoculation, without inoculation, and on metabolites of *F. verticillioides* and *P. oxalicum* which had been diluted as the calibration basis showed the same and consistent fluorescence emission wavelength.

The difference of maize extract media concentration gave effect on the amount of available nutrition for *F. verticillioides* and *P. oxalicum* in producing metabolites. The smaller the concentration of maize extract media, the less the detected of *F. verticillioides* and *P. oxalicum* metabolites emission wavelength on the test samples. *F. verticillioides* and *P. oxalicum* metabolites in maize extract media concentration of 25% were detected each as many as two and three test samples. Moore-Landecker, (1972) pointed out that carbon is an essential element for fungi because fungi require carbon element in a massive amount compared to other essential elements and carbon is main nutrition for fungi.

*F. verticillioides* and *P. oxalicum* detection on maize seeds using fiber optic fluorescence spectroscopy method showed the accurate result with 24 hours incubation period. This method is faster than blotter method which is a standard method of seed-borne fungi detection. Blotter-method needs 7 days incubation period. Based on the result, fiber optic fluorescence spectroscopy is able to be developed as seed borne pathogenic fungi detection (Mathur and Kongsdal, 2003).

**D. Metabolite Compound Analysis:** Analysis using Py-GCMS showed fumonisin B1 as the main compound (58.87%) on *F. verticillioides* metabolite. Fumonisin B1 is mycotoxin produced by 11 species of Fusarium Fungi, including *F.verticillioides* which is from maize (Guatema *et al*., 2000). Fumonisin B1 is a main contaminant of maize and becomes a concern because there is an epidemiology correlation between consumption of fumonisin-contaminated maize and esophagus cancer also neural tube defects in some people. Fumonisin is a diester compound of propane-1,2,3-tricarboxylic acid with the 2-amino-12,16-dimethyl-3,5,10,14, 15-pentahydroxy group having a molecular weight of 721 with the molecular formula $C_{34}H_{59}O_{15}$. The properties of fumonisin are hygroscopic, water-soluble, stable in methanol at storage -18°C, but easily degraded at 25°C or higher. Phuong *et al*., (2015) pointed out that fumonisin produces a wavelength of 500 nm when excited with a wavelength of 420 nm. Ali *et al*., (1998) had succeeded isolating *F. verticillioides* on maize and detecting fumonisin B1 on maize from Yogyakarta, Purworejo, and Surakarta with fumonisin concentration of 0.016-2.24 mg/kg.

Py-GCMS showed that ochratoxin A as the main compound (73.40%) in *P. oxalicum* metabolite. Ochratoxin is mycotoxin produced by secondary metabolism of many filament species of the genera Penicillium. Chemically, ochratoxin A is a mixture of pure and colorless crystals which has chemical formula $C_{20}H_{18}ClNO_6$ with a molecular weight of a 403.8 g/mol and produces a wavelength of 523 nm when excited with a wavelength of 480 nm (Zhenzhen *et al*., 2010). This molecule has green fluorescence under violet light (Khoury and Athoi, 2010). Ochratoxin A is a potential and important toxins like aflatoxin. The main organ target of

okratoksin is kidney and is known as nephrotoxin in all animal species and human (Creppy, 1999).

## CONCLUSIONS

Fluorescence emission wavelength of *F. Verticillioides* and *P. oxalicum* metabolites incubated on maize extract media for 24 hours can be measured using the fluorescence spectroscopy method. Based on fiber optic fluorescence spectroscopy. *F. verticillioides* is able to be detected at the emission wavelength of 505 nm, on the other hand, *P. oxalicum* at the emission wavelength of 552 nm. The main metabolite compounds of *F.verticillioides* metabolite is fumonisin B1 (58.87%) and *P. oxalicum* metabolite is ochratoxin A (73.40%).

## SUGGESTIONS

Fungi detection using fiber-optic fluorescence spectroscopy method needs to be developed in order to find out fungi detection-method on seeds in large quantities simultaneously.

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