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Original Research Paper

Analysis of the Protein Profile of Cassava Plantlets (*Manihot esculenta* Crantz.) Resistance to Fusarium Wilt Disease

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Abstract: Fusarium wilt disease is still a production constraint in Cassava (*Manihot esculenta* Crantz.) cultivation. The disease is caused by the fungus *Fusarium oxysporum* (*Fo*), which to date has not been cured. It is expected that the use of varieties of cassava that are resistant to Fusarium wilt is an important alternative for disease control. Induced Resistance cassava research on Murashige and Skoog medium containing Fusaric Acid (FA) selective concentration has been done before and there were indications FA concentration tolerant to the selection of resistant plantlets *in vitro*. It is hoped that cassava plantlets that are resistant to FA will also be resistant to *Fo*. In previous studies, the inoculation of *Fo* fungal isolates on resistant cassava plantlets was carried out *in vitro*, followed by DNA pattern analysis compared to controls. The results of the DNA pattern analysis, in the form of a new (specific) DNA band that has a size of 550 bp (OPA_1) and 300 bp (OPA_10), are predicted to be candidates for RAPD markers for cassava resistance to *Fo*. Based on the results of these previous studies, it is necessary to study more deeply in this study to ascertain whether the new DNA strand is really a peroxidase protein that causes cassava plantlets to be resistant to *Fo* by protein profile analysis. The results of protein profile analysis showed that the appearance of new protein bands (around 98 kD) indicated the formation of PR-protein (peroxidase) in cassava plantlets that were resistant to *Fo* and missing protein bands (around 65 kD) in cassava plantlets.

Keywords: Fusaric Acid, *Fusarium oxysporum*, *In Vitro*, *Manihot esculenta*, Protein Profile

Introduction

Cassava (*Manihot esculenta* Crantz.), is the third most important crop in the world and a source of food and tree income throughout the tropics. Cassava cultivation can provide sustenance to more than 500 million farmers (Eleazu *et al*., 2014; Amponsah *et al*., 2014). Cassava is an important food commodity in Indonesia and in the future this commodity will have a more strategic role in the lives of the people and the country's economy. Based on the area of harvest of food commodities, cassava ranks third after rice and corn, which are the three main sources of carbohydrates in the community (Fauzi *et al*., 2015). According to FAOSTAT (2019), Indonesia is the 6th cassava producing country in the world after Nigeria, Congo, Thailand, Ghana and Brazil with a production of 14,586,693 tonnes. The center of cassava land in Indonesia is controlled by Lampung Province with production reaching 7,387,084 tonnes. This situation makes Lampung a third supplier of national cassava production from the national production of 21,801,415 tonnes (Badan Pusat Statistik, 2015).

One of the problems encountered in cultivating cassava is Fusarium wilt caused by the fungus *Fusarium oxysporum*. Arinze (2005) and Okigbo *et al*. (2009) reported that 50% of cassava tubers produced and harvested in Nigeria were lost due to disease. The main causes of decay of cassava include: *Aspergillus flavus*,

Aspergillus niger, *Botryodiplodia theobromae*, *Collectotrichum* spp., *Geotrichum candidum*, *Penicillium chrysogenum*, *Pennicillium digatum* and*Fusarium oxysporum* (Ogunleye and Ayansola, 2014; Raphael*et al*., 2015; Gwa *et al*., 2015). This organism reduces the quantity and quality of the plant tubers (Amusa *et al*., 2003).

One alternative way of controlling disease that is safe for the environment, among others, is using resistant varieties. The development of cassava varieties that are resistant to *Fusarium oxysporum* can be carried out by *in vitro* selection methods, namely culturing explants in the form of tissues or organs on medium containing selective concentrations of fusaric acid (Nurcahyani, 2017; Nurcahyani *et al*., 2016a; 2016b; 2019a; 2019b; 2020).

Fusaric acid is a metabolite produced by several species of fungi from the genus Fusarium. This acid can be toxic (concentrations more than 10^{-5} M) thus inhibiting growth and culture regeneration, but at non-toxic concentrations (below 10^{-6} M) it actually helps to induce phytoalexin synthesis, a plant response forms to inhibit pathogenic activity (Bouizgarne *et al*., 2006).

The use of fusaric acid as a selective agent in *in vitro* selection can produce mutant cells or tissues that are insensitive to fusaric acid, so that after being regenerated into plants, they can produce strains resistant to pathogenic infections. The identification of mutants or variants that are insensitive to fusaric acid by *in vitro* selection has been carried out, among others, on the *Sphatoglottis plicata* (Nurcahyani *et al*., 2016a; 2016b), vanilla (Nurcahyani *et al*., 2012; 2014; Nurcahyani, 2017), Cassava (Nurcahyani *et al*., 2019a; 2019b), *Phalaenopsis amabilis* (L.) Bl. (Nurcahyani *et al*., 2020).

Research on Induced Resistance cassava with fusaric acid has been conducted before and found indications of tolerant fusaric acid concentrations for the selection of resistant plantlets *in vitro*. Inoculation of *Fusarium oxysporum* (*Fo*) fungal isolates on resistant cassava plantlets was carried out *in vitro*, followed by DNA pattern analysis compared to controls. The output of this study, in the form of a cassava mutant with a new (specific) DNA band measuring 550 bp (OPA_1) and 300 bp (OPA_10), is predicted to be a candidate RAPD marker for cassava resistance to *Fo*. Based on the results of these studies, further study needs to be done to ascertain whether the new DNA strand is really a peroxidase protein that causes the cassava mutant to be resistant to *Fo*, namely by protein profile analysis. The special characteristics of cassava plantlet and related to resistance to fusarium wilt can be examined molecularly, that is, through the analysis of protein profiles using the SDS-PAGE method. Comparison of protein bands formed by electrophoretic separation can be carried out to identify the gene product produced during *Manihot esculenta* plantlets selected by fusaric acid.

Based on the above background, more in-depth research is needed on the Induced Resistance of cassava plantlet resistant to Fusarium wilt disease, so that ultimately the long-term goal of this research will be achieved, namely the acquisition of cassava mutant varieties that are resistant to *Fusarium oxysporum.*

Materials and Methods

The tools and materials used in this research include Laminar Air Flow Cabinet (LAF), autoclave, freeze dryer, centrifuge, 0.2 and 1.5 mL microtube, shaker, electrophoresis tank, spectrophotometer, hot plate, microwave, PCR machine, UV transilluminator; Cassava plantlet (*Manihot esculenta* Crantz.), pure fusaric acid produced by Sigma chemical Co. {Fusaric acid (5-butylpicolinic acid) from Giberella fujikuroi}, 70% alcohol, solid Murashige and Skoog (MS) medium, Phosphate Buffer Saline (PBS), Bovin Serum Albumin (BSA), Bio-rad dye, reagent for SDS-PAGE, the protein ladder.

Planting and Selection of Cassava Plants (Manihot esculenta Crantz*.)*

Planting plants in Murashige and Skoog (MS) medium in culture bottles and added with Fusaric Acid (FA) with concentrations of 0 ppm (control), 60, 80, 100 and 120 ppm. Each concentration was carried out 5 times and each replication consisted of 2 cassava plants in each culture bottle. Plant selection was carried out for 30 days. At the end of the fourth week he was assessed to determine the concentration of fusaric acid yielding LC_{50} for *in vitro* cassava crop selection.

Cassava Plantlets Protein Extraction

Protein extraction was carried out by counting 1 g of plantlet leaves with each added 300 µL of Phosphate Buffer Saline (PBS) (8.55 g/L NaCl, 1.33 g/L $Na_2HPO_4.2H_2O$, 0.34 g/L $NaH_2PO_4.H_2O$. L) with a pH of 7 as extraction buffer and added with a protease inhibitor, then crushed using a mortar and pestle until homogeneous. The crushed sample was centrifuged at 13,000 rpm for 2 sec. Supernatant containing crude protein was taken and stored at -20°C (Maniatis *et al*., 1982).

Measurement of Protein Concentration

After the crude protein is obtained, measurement of the protein concentration in each sample is carried out. Protein concentration was determined using the Bio-rad method (Bio-rad Assay). Determination of protein concentration was carried out by taking 2 µL of protein samples using a micropipette plus 200 µL of Bio-rad dye and 798 µL of distilled water, then mixed by resuspension, then read by a spectrophotometer (Beckman, DU-65) at wavelength (OD 595 nm). The

protein concentration is known through the equation of the standard BSA protein standard curve function (Maniatis *et al*., 1982).

Determination of the Molecular Weight of a Protein

Determination of the Molecular Weight (MW) of protein was carried out using the SDS-PAGE method according to Maniatis *et al*. (1982). Electrophoresis at a voltage of 100 volts is carried out for 1.5 to 2.5 h. Protein staining was carried out in a 0.10% solution of Coomasie Brilliant Blue, shaken with a shaker overnight. After staining, destaining is carried out to remove excess color by immersing the gel in a destaining solution (50 mL distilled water, 40 mL methanol, 10 mL glacial acetic acid) until the gel becomes clear with separate bands from each other. The gel was then stored in 10% glacial acetic acid and then dried with a plate kit. The protein bands formed in the gel after electrophoresis were determined by their molecular weight (kD). The molecular weight of the sample protein at each migration distance is obtained by extrapolating every distance of the desired sample protein band at the 2 migration distance of the protein marker bands that flank the sample protein band in question, so that a log of molecular weight is obtained, then the molecular weight of the protein band in question can be known.

To detect the presence of new (specific) proteins, it was done by comparing the protein profiles of Cassava leaflets that were not affected by fusaric acid with cassava plantlets induced by fusaric acid.

Results and Discussion

In this study, using cassava plantlet (*Manihot esculenta* Crantz.) *In vitro* and treated with various levels of fusaric acid. The fusaric acid used in this study contained five different concentration levels, namely 0 ppm (control), 60, 80, 100 and 120 ppm. The results of the selection of cassava plantlets that have been induced using fusaric acid with various different concentration levels are presented in Fig. 1.

Based on Fig. 1, visually, the plantlets in each treatment varied, especially seen from the poor morphological characters of the plantlets, namely small size, shoots and roots that were slightly formed and some were not formed at all. The visual appearance of the plantlets which were originally green turned pale green and brown (browning) after being treated with fusaric acid In browning-resistant planlets it occurs only at the base of the planlet in contact with the medium and does not extend to the ends. Plantlets that do not withstand browning quickly expand to all parts of the plantlet.

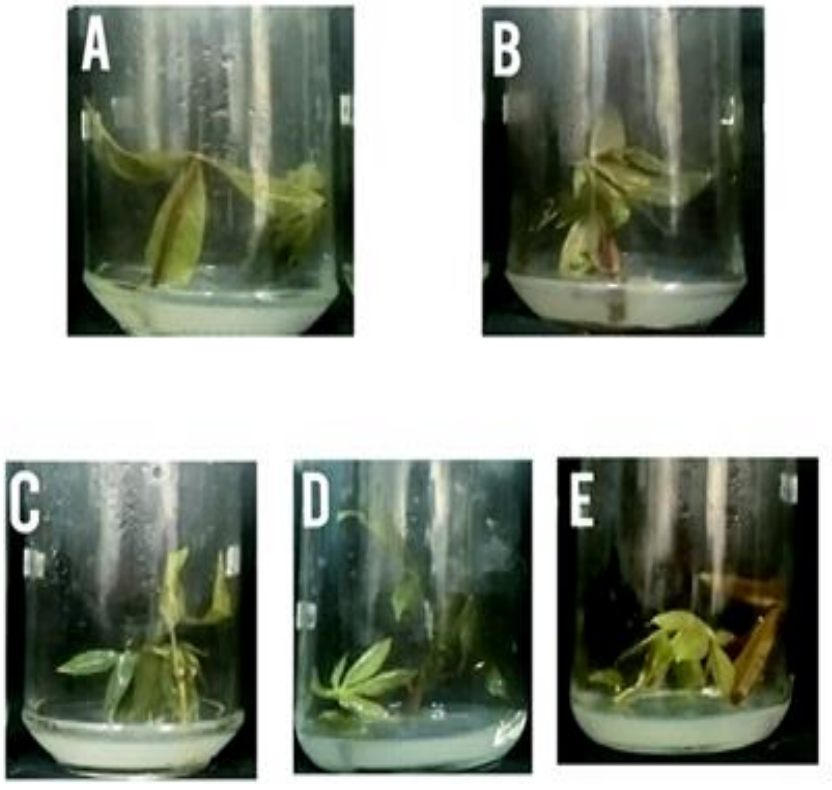

Fig. 1: Cassava plantlets with various concentrations of fusaric acid (A) 0 ppm “Control”, (B) 60 ppm, (C) 80 ppm, (D) 100 ppm and (E) 120 ppm

Kuźniak (2001) stated that fusaric acid can affect the elimination of O_2^- through quinone reduction which induces brown color and is thought to be associated with phenol transformation. The results of the phenol oxidation reaction are brown, so that the plantlets that have undergone phenol oxidation appear brown. If this oxidation reaction takes place continuously, the brown color will spread and diffuse into the medium and affect the growth of other plantlets that are cultured with the browning plantlets.

The specific character of Cassava plantlets and related to resistance to *Fusarium oxysporum* can be examined molecularly, namely through protein profile analysis using the SDS-PAGE method.

In principle, genes (DNA fragments) are transcribed into mRNA in the cell nucleus. Furthermore, the triplet base codon in mRNA is translated by the ribosome into amino acids. From this set of amino acids, a specific protein is formed. If there is a change in base and or structure in DNA/RNA, it is called a mutation which can be in the form of addition, deletion and substitution. As a result of this change in base, the expressed protein would certainly be different from those that did not undergo mutation. This concept will be discussed in Cassava's research with fusaric acid stress for resistance to *Fusarium oxysporum*. Plants treated with Fusaric acid will activate genes, including peroxidase, glucanase and chitinase genes (Saravanan *et al*., 2004).

The protein profile was obtained after the crude extract protein (concentration about 10 µg) was running by electrophoresis in 1D vertical gel (SDS-PAGE) for 2 h, with a voltage of 90 volts. The protein banding pattern formed in the mutant candidates turned out to be different bands or bands compared to the control. This occurred in all samples both at fusaric acid treatment concentrations of 60, 80, 100 and 120 ppm (Fig. 2).

Based on Fig. 2, it was found that the expression of a new protein band with a molecular weight of about 98 kD and a missing protein band with a molecular weight of 65 kD was found in the cassava *Manihot esculenta* Crantz. plantlet treated with 120 ppm fusaric acid stress. From the protein profile analysis, it indicated that *M. esculenta* plantlets treated with 120 ppm fusaric acid gave a different band than the control and fusaric acid treatment with a concentration of 60, 80 and 100 ppm.

This shows that fusaric acid triggers the expression of peroxidase genes in the Cassava plantlets, so that their activity is higher and the protein bands are more clearly stained. Based on this, it is suspected that there has been a mutation in the promoter so that the band with molecular weight approximately 98 kD can be indicated as a marker for *Fusarium oxysporum* resistant cassava plantlets.

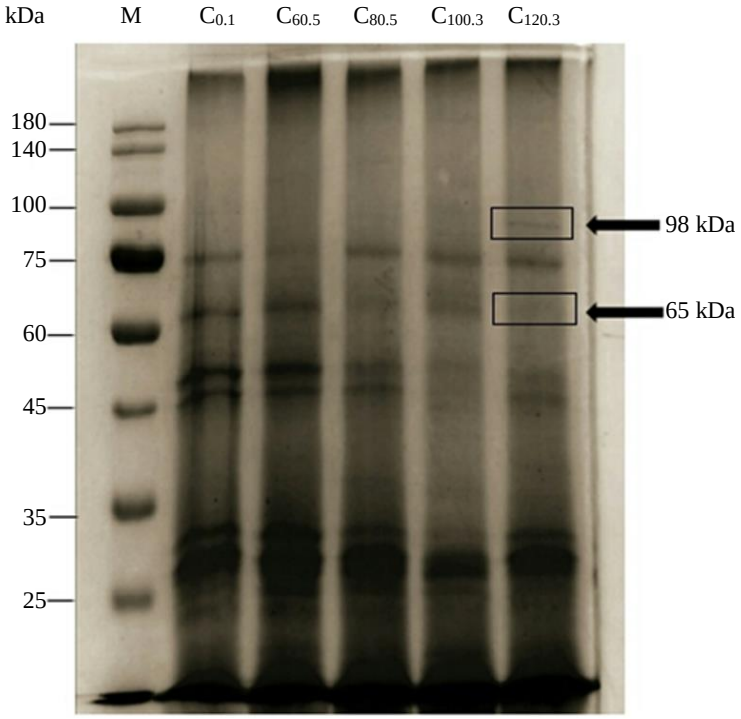

Fig. 2: Protein profile of cassava leaves (*Manihot esculenta* Crantz.) Induced by fusaric acid using SDS-PAGE 1D method. M = Marker. $C_{0.1}$ = control, $C_{60.5}$ = 60 ppm, $C_{80.5}$ = 80 ppm, $C_{100.3}$ = 100 ppm, $C_{120.3}$ = 120 ppm. The arrow () = indicates a new protein band formed (approximately 98 kD) and disappeared (approximately 65 kD) in cassava plantlets that are resistant to *Fusarium oxysporum*

According to Gunanti *et al.* (2010), the protein band thickness of the SDS-PAGE results illustrates the high and low concentration of a protein contained in the test sample. The induced resistance of cassava plantlets with fusaric acid treatment, one of the possibilities was due to the activation of the peroxidase gene which encodes the peroxidase enzyme and plays an important role in resistance to *Fusarium oxysporum*.

Research conducted by Ye and Ng (2002) on French beans has successfully isolated protein peroxidase and produced bands with molecular weight approximately 37 kD. In sorghum plants infected with *Fusarium moniliforme*, induction of resistance protein with molecular weight around 18 and 30 kD and predicted as protein peroxidase (Kumari *et al*., 2006). A protein with molecular weight around 18.9 kD has also been found by Ye and Ng (2009), which is an antifungal induction protein from Japanese takana seeds (*Brassica juncea* var. *integrifolia*). Meanwhile, Ye *et al*. (2011), found an antifungal protein with molecular weight around 30 kD in red cabbage (*Brassica oleracea*). Nurcahyani *et al.* (2016a), in their research on vanilla (*Vanilla planifolia*) which was induced by fusaric acid and infected with *Fusarium oxysporum*, causing resistance induction with a molecular weight of about 18 kD and predicted as protein peroxidase.

Genes need the right time and conditions to be expressed in the cycle of growth and development of plants. Entering a new stage of development, plants need the expression of several genes to produce proteins that play a role in every metabolic reaction in cells. In this case, it may be endogenously, the gene encoding the peroxidase enzyme is present in plantlets, but is only expressed when the condition is stressful, in this case fusaric acid. The expression of this peroxidase enzyme appears as a mechanism of resistance to fusaric acid stress and is also a resistance to Fusarium (Bouizgarne *et al*., 2006).

Conclusion

The character of cassava (*Manihot esculenta* Crantz.) plantlets which are resistant to *Fusarium oxysporum* can be proven molecularly, namely through protein profile analysis. The protein band (molecular weight approximately 98 kD) on SDS-PAGE 1D indicated the resistance to *M. esculenta* plantlets against Fusarium wilt disease. The protein with a molecular weight of about 98 kD is predicted to be a protein peroxidase, which plays a role in resistance to *F. oxysporum*. The perspective to ensure that the protein bands with molecular weight of about 98 kD is protein peroxidase, it is necessary to isolate the protein, analyze its amino acid base by aligning it with the protein peroxidase sequence of other species, analysis of transient gene expression and/or Western blot analysis, this will be done at the next research stage.

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Author's Contributions

Endang Nurcahyani: Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Hardoko Insan Qudus: Coordinated the data analysis and translation of manuscript.

Ferina Evlin: Analyze protein profile data.

Ethics

The corresponding author confirms that all author have read and agree to the publish version of the manuscript. This article is original and contains unpublished material and no ethical issues involved.

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