Journal of Plant Sciences
2016; 4(5): 102-105
http://www.sciencepublishinggroup.com/j/jps
doi: 10.11648/j.jps.20160405.12
ISSN: 2331-0723 (Print); ISSN: 2331-0731 (Online)

The Protein Profile of the Plantlets of *Spathoglottis plicata* Bl. Induced Resistance to *Fusarium oxysporum*

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To cite this article:
Endang Nurcahyani, R. Agustrina, T. T. Handayani. The Protein Profile of the Plantlets of *Spathoglottis plicata* Bl. Induced Resistance to *Fusarium oxysporum*. *Journal of Plant Sciences.* Vol. 4, No. 5, 2016, pp. 102-105. doi: 10.11648/j.jps.20160405.12

Received: July 18, 2016; **Accepted**: August 4, 2016; **Published**: August 21, 2016

Abstract: Ground orchid (*Spathoglottis plicata*) cultivated have many constraints such as the appearance of fusarium wilt disease caused by *Fusarium oxysporum* (*Fo*). A resistant *S. plicata* plantlet to *Fo* has been initiated by *in vitro* selection on Vacin and Went (VW) medium containing fusaric acid (FA) at concentrations of 10 ppm, 20 ppm, 30 ppm, and 40 ppm, compared with controls (0 ppm), and there were indications of FA tolerant of the selected plantlets. This study aims to describe the formation mechanism of Induced Resistance by analysing the protein profile of the plantlet. The protein profile was analysed by SDS-PAGE methods. The results showed that protein profiles of explants leaves was significantly different from the control. There was an initiation of a new protein bands (± 19 kD) indicating the formation of PR-protein (peroxidase) on the plantlets which responsible for *S. plicata* resistant to *Fo*.

Keywords: Protein Profile, *Spathoglottis plicata* Bl., In Vitro, Induced Resistance, *Fusarium oxysporum*

1. Introduction

One of the problems commonly encountered in growing ground orchids (*Spathoglottis plicata*) is a fusarium wilt. This ailment is caused by a pathogenic fungus, *Fusarium oxysporum* (*Fo*), that infect several parts of plants such as stems, leaves or roots.The disease can lead to plant death and the losses of more than 50% and is difficult to control by simply using a fungicide [14].

The development of resistant cultivars of *S. plicata* can be done for example by in vitro selection method that explants of tissue or organ in the form of medium containing fusaric acid concentrations selectively [3]. Some parameters can describe the mechanisms of plant resistance to pathogen infection include an increase in phenolic compounds, an increase of peroxidase (including a group of PR-proteins), and the lignifikasi [1, 8, 13].

In plants treated with FA, activates genes among genes peroxidase [12]. Comparison of protein bands formed by electrophoretic separation can be carried out to identify the gene product produced during plantlets *S. plicata* selected using FA. One-dimensional protein electrophoresis method with Sodium Dodecyl Sulphate-Polycrylamide Gel Electrophoresis (SDS-PAGE) is one method to analyze proteins with separate bands of protein in the sample based on their molecular weight [9].

Resilience affected are not specific for the pathogen, so it can be more efficient in its implementation [2]. Peroxidase (including PR-protein group) is one of the compounds that can describe mechanisms of plant resistance to pathogen infection [13]. PR-protein is a protein that is released by plants in response to several compounds (inducer). Plant infection by pathogens such as fungi, bacteria, and viruses can cause the formation of PR-protein [4, 11]. Synthesis and accumulation of PR-proteins have very important role in plant defense against pathogens. This has been identified on the roots of banana plants infected by *Fusarium oxysporum* f. sp *cubense* [4, 12], and vanilla infected by *F. oxysporum* f. sp. *vanilla* [10]. Fusaric acid is known to be the result of secondary metabolites produced by the fungus *Fusarium heterosporum* Nee. and one of the toxin that is responsible the incidence of wilt symptoms in some plants [7]. Fusaric Acid is non-toxic concentrations (below 10^{-6} M) can induce phytoalexin synthesis, a form of response to inhibit the activity of plant pathogens. Through this method has done a lot of research and has secured the nature of resistance to diseases such as Fusarium wilt in banana, wheat, and carnations. The use of FA as a selective agent in vitro

selection of cells or tissue can produce mutant insensitive to the FA, so that after the regenerated into plants can produce resistant strains of infectious pathogens [3]. Comparison of protein bands formed by electrophoretic separation can be carried out to identify the gene product produced during *S. plicata* plantlets selected by FA.

2. Materials and Methods

The tools used in this study include aluminum foil, Autoclave, Laminar Air Flow Cabinet (LAF)-ESCO brand, scalpel, scalpel blades, filter paper, micropipette, pipette tip, spectrophotometry (Shimudzu UV 800), test tubes, test tube rack, and Canon Ixus 951S.

The materials used in the research include *Spathoglottis plicata* plantlets sterile in a culture bottle age 2 months obtained from a private collection (Dr. Endang Nurcahyani, M.Si.), pure fusaric acid produced by Sigma chemical Co. {Fusaric acid (5-butylpicolinic acid) from Giberella fujikuroi}, 70% alcohol, distilled water, Benzine Amino Purine (BAP), Hydrochloric acid (HCl), and chemicals VW medium (Vacin & Went) solid.

Protein extraction was performed according to [9]. Protein extraction is done by weighed 1 g leaves of plantlets and each plus 300 mL Phosphate Buffer Saline (PBS) (NaCl 8.55 g/L, $Na_2HPO_4.2H_2O$ 1.33 g/L, $NaH_2PO_4.H_2O$ 0.34 g/L) at pH 7 as the extraction buffer plus protease inhibitors and then crushed using a mortar and pestle until homogeneous. Samples were centrifuged at a speed of 13,000 rpm for 2 seconds. The supernatant containing the crude protein then removed and stored at -20°C.

After the crude protein is obtained, measurement of protein concentration in each sample. The protein concentration was determined using the Bio-rad Assay. Bovin Serum Albumin (BSA) was used as a standard to calculate the concentration of protein. As a blank used 200 mL of Bio-rad dye and 800 mL of distilled water. Determination of protein concentration is done with captured 2 mL protein sample using a micropipette plus 200 mL of Bio-rad dye and 798 mL of distilled water, then mixed in a way resuspended, then read with a spectrophotometer (Beckman DU-65) at the wave length (OD 595 nm). The concentration of proteins known to function through the standard curve equation BSA protein standard [9].

The determination of Molecular weight (Mw) of proteins made by using SDS-PAGE according to [9]. Resolving gel 12% gel is inserted into a mold and wait a few moments until the gel is polarized. Stacking gel 6% inserted above the resolving gel and a comb to make the wells fitted insert a protein sample. After stacking the polarizing gel, the gel is removed from the mold and plate coupled with electrophoresis apparatus. Running buffer of 0.1% was poured into a tub and a removable comb. Then the samples were taken 10 ml protein was mixed with 2 ml of sample buffer. For the marker, taken 10 ml protein marker (Fermentas) was mixed with 2 ml of sample buffer. All solutions are heated in boiling water for 2 minutes and immediately cooled in crushed ice. Furthermore, the sample solution marker protein and protein incorporated into wells in the gel (12% acrylamide gel). Electrophoresis at a voltage of 100 volts performed for 1.5 to 2.5 hours.

Staining of protein bands was done by soaking the gel electrophoresis results in a solution of 0.10% Coomasie Brilliant Blue shaken by shaker overnight. Once colored, destaining performed to remove excess color by soaking the gel in destaining solution (50 mL of distilled water, 40 mL of methanol, 10 mL of glacial acetic acid) until the gel became clear with ribbon isolated from one another. Gels are then stored in 10% glacial acetic acid and then dried with plate kit. Protein bands were formed in the gel after electrophoresis determined molecular weight [9].

Molecular weight (Mw) of protein samples at each migration distance is obtained by extrapolating each distance protein band samples desired at a distance of migration 2 protein band markers flanking protein band samples in question, in order to obtain log Mw, the Mw protein band in question can be known [5]. To detect the presence of a new protein band (specific) was done by comparing the protein profile of *S. plicata* plantlets control with *S. plicata* plantlets were scanned of FA concentrations of 10, 20, 30, and 40 ppm.

3. Results and Discussions

In principle, the genes (DNA fragments) is transcribed into mRNA in the nucleus of the cell (nucleus). Furthermore, the base of triplet codon on the mRNA is translated by ribosomes into amino acids. From the set of amino acids is then formed a specific protein. If there are changes in the structure of the base and or DNA/RNA is called a mutation that can be an addition, deletion, and substitution. As a result of changes in the base, the protein expressed will of course be different to those without the mutation. This concept will be discussed in the research of *S. plicata* with FA for stress resistance to *Fo*. Plants that was treated with the FA, will activate genes include peroxidase genes, glucanase and chitinase [12].

Protein profiles obtained after crude protein extract (concentration of approximately 10 g) in running the 1D gel electrophoresis in a vertical (SDS-PAGE) for 2 hours, with a voltage of 90 volts. Protein banding pattern formed on the candidate mutants there was a different band compared with controls. This happens on all samples well on FA treatment concentrations of 10, 20, 30, and 40 ppm (Figure 1).

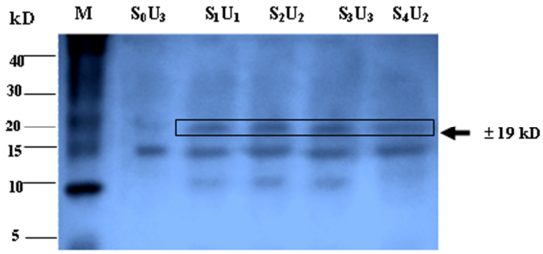

Figure 1. *Protein profiles of plantlets of S. plicata induced resistance to Fusarium oxysporum by using SDS-PAGE 1D.M = Marker. S_0U_3 = control, S_1U_1, S_2U_2, S_3U_3, S_4U_2 = S. plicata plantlets induced resistance of FA 10 ppm, 20 ppm, 30 ppm and 40 ppm. New protein band that formed in S. plicata plantlets induced resistan to Fo (± 19 kD) indicated by arrow.*

Based on Figure 1, there was an expression of the protein bands were colored thicker with Molecular weight (Mw) of approximately 19 kD in *S. plicata* plantlets were treated with FA stress. From the analysis of the protein profiles indicate that the plantlets of *S. plicata* who were treated FA concentrations of 10, 20, 30, and 40 ppm gives a different band than the untreated FA.

In Figure 1, can be visualized clearly the protein bands were smeared thicker (Mw ± 19 kD) in the FA treatment with various concentrations (10, 20, 30, and 40 ppm), compared with controls. This shows that the FA trigger existing peroxidase gene expression in *S. plicata* plantlets, so the higher activity and the protein bands smeared clearer. Based on this, alleged that there has been a mutation in promoterso that the ribbon with Mw ± 19kD can be indicated as a marker for resistant *S. plicata* plantlets toward to *Fo*. Induced resistance of *S. plicata* plantlets to the treatment of FA, one possibility is caused by activation of the peroxidase gene that encodes the enzyme peroxidase and play an important role in resistance to *Fo*. It can be associated with a peroxidase enzyme activity that is increasing in line with increased concentrations of FA.

Research conducted by [15] on French bean peroxidase protein has been isolated and produced by Mw37 kD band. In sorghum infected with *Fusarium moniliforme*, resulting in the induction of resistance to the Mw protein 18 kD and 30 kD, and is predicted to be protein peroxidase [16]. Protein with Molecular weight 18.9 kD has also been found by [6], an antifungal protein from the seeds of Takana Japan (*Brassica juncea* var. *integrifolia*). While [17], found the antifungal protein with Mw 30 kD on red cabbage plant (*Brassica oleracea*).

Gen takes time and the right conditions to be expressed on a cycle of growth and development of plants. Entering a new phase of development, plants require the expression of several genes to produce proteins that play a role in all metabolic reactions in the cell. In this case it may be endogenous peroxidase genes encoding enzymes that exist in planlet of *S. plicata*, but only expressed when in a state gripped, in this case the FA. Peroxidase enzyme expression is emerging as one of the mechanisms of resistance to stress FA and also the resistance to the Fusarium [3].

4. Conclusion

Characters *Spathoglottis plicata* plantlets which are resistant to *Fusarium oxysporum* can be proved, among others, through molecular analysis of protein profiles. The new protein bands (Molecular weight ± 19 kD) on SDS-PAGE 1D indicate the formation of *S. plicata* plantlets resistance to *Fusarium oxysporum*. Proteins with a molecular weight of approximately 19 kD protein is predicted a peroxidase, which plays a role in resistance to *Fusarium oxysporum*.

Acknowledgement

This research was supported by the grant of Research Grantsand Community Services in Higher Education 2015 Batch 1 Programmed, Skim of Fundamental, *BOPTN* Lampung University, Bandar Lampung, Indonesia, with Contract No. 158/UN26/8/LPPM/2015. Tanggal 30 Maret 2015. Thanks for Ministry of Research, Technology and Higher Education, Republic of Indonesia.

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