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Analysis of protein profile and reducing sugar content of cassava plants (*Manihot esculenta* Crantz) results of induced resistance with salicylic acid

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Abstract

Cassava (*Manihot esculenta* Crantz) is an important food commodity in Indonesia, and in the future this commodity will play an increasingly strategic role in people's lives and the country's economy. But until now there are still many obstacles, one of which is fusarium wilt disease. Fusarium wilt disease can be controlled by using superior cultivars that are resistant to the disease, by applying salicylic acid. The aim of this study was to analyze the protein profile and reducing sugar content in cassava plants after being induced with salicylic acid and compare them with controls. This research used a Completely Randomized Design (CRD) with one factor, namely the addition of salicylic acid consisting of 5 concentration levels, namely 0 ppm, 80 ppm, 100 ppm, 120 ppm, and 140 ppm. Each concentration was repeated 5 times. This research data is in the form of qualitative data and quantitative data. Qualitative data is presented in comparative descriptive form and presented in the form of photographs. Quantitative data was tabulated with different concentration factors and analyzed using Analysis of Variance then further tested with the Honestly Significant Difference Test (BNJ) at the 5% level. The research results showed that there was a missing protein band, namely a band at a molecular weight of 115, and there was also the appearance of a new band, namely a molecular weight of 85 kDa, as well as an increase in the reducing sugar content in cassava plants along with increasing salicylic acid concentrations.

Keywords : Salicylic acid; Cassava; Protein profile; Reducing sugar; Induced resistance**.**

1. Introduction

Cassava (*Manihot esculenta* Crantz) is generally grown by Indonesian farmers because it is an important food as a source of carbohydrates besides rice and corn [1]. Cassava is an important food commodity in Indonesia, and in the future this commodity will play an increasingly strategic role in people's lives and the country's economy [2]. During its growth, cassava plants experience serious problems such as disease from pathogenic fungi, bacteria or viruses. The pathogenic fungus that often attacks cassava plants is *Fusarium* sp. [3].

The use of superior varieties through *in vivo* selection with the addition of salicylic acid is an efficient alternative method of disease control. Salicylic acid is a resistance-inducing agent that is known to be used to control plant pathogens [4]. Salicylic acid is a simple phenolic compound that plays an important role in regulating physiological processes and plant immune responses. The use of salicylic acid as a signal transduction in plant defense networks has been observed and characterized in a number of genes that function in salicylic acid biosynthesis [5]. Pathogenic infection increases salicylic acid levels and promotes transcription of genes encoding pathogenesis-related proteins in plants, thereby conferring disease resistance. Proteins play an important role in the structure and function of all living cells [6]. In

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principle, genes (DNA fragments) are transcribed into mRNA in the cell nucleus (nucleus). Next, the triplet of base codons in this mRNA is translated (translated) by ribosomes into amino acids. From this group of amino acids, a specific protein is then formed. If there is a change in the bases and/or structure of DNA/RNA, it is called a mutation, which can be in the form of addition, deletion and substitution. As a result of these base changes, the protein expressed will certainly be different from one that does not experience the mutation [7]. Plants treated with fusaric acid (FA) will activate genes including peroxidase, glucanase and chitinase genes.

In addition, the level of salicylic acid administration also affects the concentration of reducing sugars. The higher the concentration of salicylic acid given, the greater the concentration of reducing sugar produced. Sugars in plants play a role in maintaining plant health by ensuring balanced sugars so that they can contribute to resistance to pathogen attack [8]. The aim of this study was to analyze the protein profile and reducing sugar content in cassava plants after being induced with salicylic acid and compared with controls.

2. Material and methods

The tools and materials used in this research were cassava plant seeds, salicylic acid, 70% alcohol, marker (Page-Ruler Low Range Unstained Protein Ladder) acrylamide/bisacrylamide solution, *Coomasie Brilliant Blue* (CBB*), Bovine Serum Albumin* (BSA), Tris-HCl buffer, *Sodium Dodecyl Sulfate* (SDS), glucose solution, Nelson's reagent, Regensia arsenomolybdate, UV-Vis spectrophotometer and electrophoresis equipment.

This research used a Completely Randomized Design (CRD) with one factor, namely the addition of salicylic acid which was divided into 5 concentration levels, namely 0 ppm, 80 ppm, 100 ppm, 120 ppm, and 140 ppm. Each of these concentrations was 5 repeated.

2.1. Salicylic Acid Treatment

Pengenceran asam salisilat dilakukan dengan melarutkan kristal asam salisilat ke dalam aquades 500 ml dalam Dilution of salicylic acid was carried out by dissolving salicylic acid crystals in 500 ml distilled water in an Erlenmayer for each concentration level of 0 ppm, 80 ppm, 100 ppm, 120 ppm, 140 ppm with 5 repetitions. The salicylic acid solution with a concentration of 0 ppm contains 100% pure distilled water 500 ml without salicylic acid crystals. A salicylic acid solution with a concentration of 80 ppm is made by dissolving 0.04 grams of salicylic acid crystals in 500 ml of distilled water, a concentration of 100 ppm is made by dissolving 0.05 grams of salicylic acid crystals in 500 ml of distilled water, then a concentration of 120 ppm is made by dissolving 0.06 grams of salicylic acid crystals. in 500 ml of distilled water, and for a concentration of 140 ppm the same thing is also done, namely dissolving 0.07 grams of salicylic acid crystals in 500 ml of distilled water. After the salicylic acid is dissolved in distilled water (homogeneous), it is then filtered with filter paper into a culture bottle. Each concentration consists of 5 culture bottles with a volume of 100 ml per culture bottle. Next, the culture bottle is covered with aluminum foil and plastic wrap

Salicylic acid treatment was carried out when the plants were 14 days old. Adding salicylic acid to cassava plants is done by pouring 50 ml of salicylic acid solution onto the stem surface for each cassava plant with various concentrations, namely 0 ppm, 100 ppm, 120 ppm and 140 ppm. Each concentration consists of 5 plants, so each concentration requires 250 ml of salicylic acid solution.

2.2. Cassava Protein Profile Analysis using the SDS-PAGE (Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis) Method

2.2.1. Extraction of cassava plant leaves

Protein extraction was carried out by counting 1 g of plant leaves with 300 µL of Phosphate Buffer Saline (PBS) added to each (NaCl 8.55 g/L, Na2HPO4.2H2O 1.33 g/L, NaH2PO4.H2O 0.34 g/ L) with pH 7 as extraction buffer and protease inhibitor added, then crushed using a mortar and pestle until homogeneous. The sample that had been crushed was centrifuged at a speed of 13,000 rpm for 2 seconds. The supernatant containing crude protein was taken and stored at 20ºC.

2.2.2. Cassava Protein Profile Analysis using the SDS – PAGE method.

After the crude protein was obtained, the protein concentration was measured in each sample. Protein concentration was determined using the Bradford protein Assay method (Bio-rad Assay). Bovine Serum Albumin (BSA) was used as a standard to calculate protein concentration. As a blank, 200µL of Bio-rad dye and 800µL of distilled water were used. Determination of protein molecular weight using the SDS-PAGE (*Sodium Dodecyl Sulphate-Polyacrylamide Gel*

Electrophoresis) method. The sequence of this model is 12% separating gel (distilled water 3.3 mL, polyacrylamide 4 mL, 1.5 M Tris pH 8.8 2.5 mL, 10% SDS 100 µL, 10% APS 90 µL) inserted into the plate pair. glass as a gel mold and wait until the gel is polarized. Stacking gel 6% (distilled water 2.6 mL, polyacrylamide 1 mL, 0.5 M Tris pH 6.8 1.15 mL, 10% SDS 50 µL, TEMED 10 µL, 10% APS 90 µL) is inserted on top of the separating gel and fitted with a comb which is used to make wells to insert protein samples.

After stacking the polarized gel, the gel is released from the mold and the plate is assembled with an electrophoresis apparatus. Running buffer 0.1% (distilled water 1 L, Tris Base 15 g, glycine 72 g, SDS 5 g) is poured into the tub and the comb is removed. Then 10 µL of protein sample was mixed with 2 µL of sample buffer. Protein and marker sample solution. Proteins were loaded into gel wells (12% acrylamide gel). Electrophoresis at a voltage of 100 volts is carried out for 1.5 – 2.5 hours. Protein bands with a higher molecular weight will form closer to the initial site of separation. Protein band staining was carried out by immersing the gel resulting from electrophoresis (the plate series was removed) into a 0.10% *Coomasie Brilliant Blue* solution and carried out using a shaker. Then, after being colored, destaining was carried out to remove the color by immersing the gel in a destaining solution (500 mL of distilled water, 40 mL of methanol, 10 mL of glacial acetic acid) so that the gel became clear with the bands separated from each other. The gel is then stored in 10% glacial acid and dried with a plate kit. The protein band formed in the gel after electrophoresis is determined by its molecular weight [9].

3. Analysis of the Reducing Sugar Content of Cassava Leaves

Analysis of reducing sugar content uses the Somogyi-Nelson method [10] with the following steps:

3.1. Making a Reducing Sugar Calibration Curve

Reducing sugar analysis is carried out by making a standard glucose solution. Glucose solution diluted to a concentration of 2mg/120 ml, 4mg/120ml, 6mg/120ml, 8mg/120ml, 10 mg/120ml, and 12 mg/120 ml was added to each test tube containing distilled water as a blank. Then, add 1 mL of reagent. Nelson (Nelson A 25 parts, Nelson B 1 part) into each tube. The solution added by Nelson was then heated for 20 minutes. The solution was cooled in a beaker to a tube temperature of 25°C, then 1 ml of regentia arsenomolybdate was added, homogenized until all the precipitate dissolved again. After homogenization again, 7 ml of distilled water was added and dissolved until homogeneous. The solution was measured with a spectrophotometer at a wavelength of 695 nm, then a calibration curve was created for the relationship between glucose concentration and absorbance.

3.2. Determination of Reducing Sugar Content

Take 1 ml of fresh cassava leaf extract at each concentration level, put it in a test tube and add 1 ml of Nelson's reagent to the tube. The solution that Nelson had added was then heated for 20 minutes. The solution was cooled in a beaker to a tube temperature of 25°C, then 1 ml of arsenomolybdate reagent was added, dissolved until the entire precipitate was homogeneous. After homogenization, 7 ml of distilled water was added and shaken until homogeneous. The solution was measured with a spectrophotometer at a wavelength of 695 nm, then a calibration curve was created for the relationship between glucose concentration and absorbance.

The reducing sugar content is calculated using formula 1 [11]:

Reducing Sugar Content (%) = (X. FP)/BS x 100%

Note:

X = Concentration (ppm)
FP= Dilution Factor
BS= Sample Weight (mg)

4. Results and discussion

4.1. Protein Profile Analysis Results

In protein profile analysis using cassava plant leaves. Leaves have a major role in transporting important elements from the roots to the leaves. Most of the identified proteins are involved in energy production and primary/secondary

metabolism in leaf tissues. In higher plants, leaves have highly specialized organs mainly seen in photosynthesis. The band pattern of the cassava plant protein profile is presented in Figure 1.

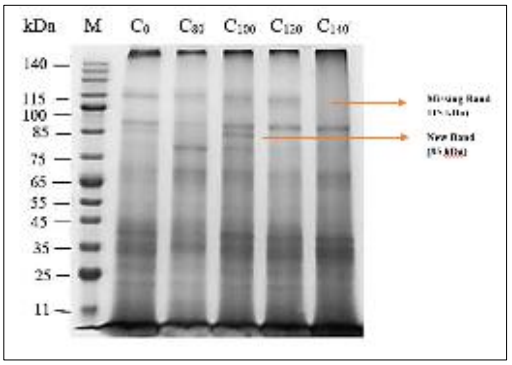

Figure 1 Ribbon pattern of cassava plant protein profile

Note :

M	: Protein markers
C_0, C_{80}, C_{100},C_{120}, C_{140}	: Protein sampels

Based on Figure 1, it can be seen that there are several sample bands that have a straight line with the marker, such as the sample band and the 65 kDa marker band. This shows that the samples have the same molecular weight because the samples used are the same, namely the leaves of the cassava plant. Molecules of the same weight will move the same distance to form a straight line. Based on the SDS-PAGE results of the protein profile of cassava (*Manihot esculenta* Crantz), there is a missing protein band, namely a band at a molecular weight of 115 kDa (treatment with a salicylic acid concentration of 140 ppm), and there is also the appearance of a new band, namely at a molecular weight of 85 kDa (treatment with an acid concentration). salicylate 100 ppm).

The thickness of the protein band resulting from SDS-PAGE describes the high or low concentration of a protein contained in the test sample. In this study, the protein bands that were seen as the thickest and clearest were the bands with molecular weights of 35 kDa and 45 kDa. Meanwhile, the protein band that consistently appears with light smear visualization is the protein band with a molecular weight of 115 kDa. The presence and thickness of the protein bands formed depend on the type, number and sequence of amino acids. This research produced several findings in the form of the disappearance of protein bands at a concentration of 140 ppm and the appearance of new protein bands at a concentration of 100 ppm. The disappearance and appearance of the protein band indicated that a mutation had occurred in the protein with salicylic acid treatment at concentrations of 100 and 140 ppm. Mutations that occur are the result of the addition of one or more nucleotides in a gene. This results in a shift in the reading frame [12].

The results of this research are supported by previous research conducted by [13] by analyzing the protein profile of cassava plants (*Manihot esculenta* Crantz) treated with fusaric acid which was then cultured in vitro. The results of this research were the appearance of a new band with a molecular weight of 98 kDa and the disappearance of a protein profile band with a molecular weight of 65 kDa. During protein synthesis, the reading of the genetic code starts from the mRNA, and the three nitrogen bases are read sequentially, therefore frame shift mutations generally cause the formation of proteins that do not function as a result of the synthesis of a new amino acid sequence from the reading of the nucleotide sequence of the mRNA which has shifted the frame. Mutations or frame shifts generally cause the formation of proteins that do not function as a result of the synthesis of a new amino acid sequence from reading the nucleotide sequence of mRNA whose frame has shifted.

Nurcahyani *et al*. [14], also conducted similar research which in his research used ground orchid plants (*Spathoglottis plicata* BI.) in vitro using fusaric acid treatment, stating that the protein profile analyzed using the SDS-PAGE method contained or appeared new protein bands at molecular weight. ±19 kDa, which is also indicated by the presence of a new protein band as the formation of PR-protein (peroxidase) in Spathoglottis plicata Bl plantlets. which shows that the S. plicata plantlets are resistant or resistant to the *Fusarium oxysporum* fungus

5. Reducing Sugar Analysis Results

Measurement of reducing sugar content on the reducing sugar standard curve. The standard curve is obtained from the comparison of reducing sugar concentration to the absorption value. The results of determining the standard curve for reducing sugar can be seen in Table 1.

Table 1 Comparison of reducing sugar concentration and absorbance

Reducing Sugar Concentration (ppm)	Absorbance (nm)
20	0,142
40	0,243
60	0,324
80	0,389
100	0,481
120	0,546

Based on the data in Table 1, the standard curve for reducing sugar is obtained, a linear equation y=0.004x +0.0686 is obtained and has a correlation value of R2= 0,995, which shows that the variation between reducing sugar concentration and absorbance is homogeneous. Figure 1.

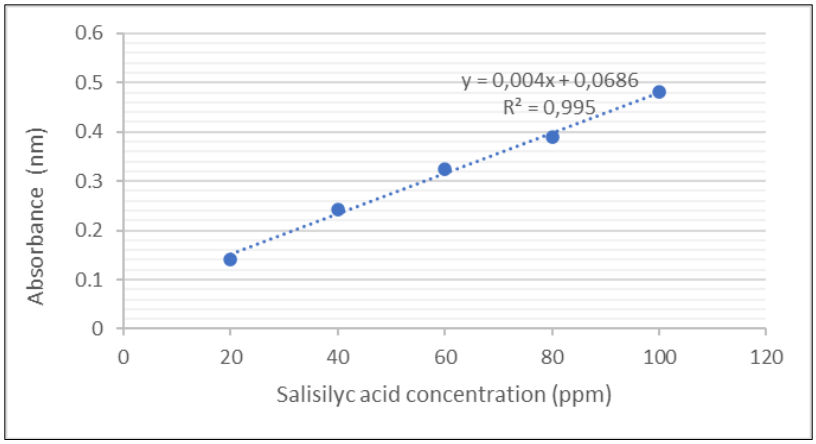

Figure 2 Reducing Sugar Standard Curve

Based on Figure 2, the reducing sugar content of each treatment can be found by substituting the absorbance value (y) of the cassava leaf extract solution sample at the maximum wavelength into the linear regression equation y = ax+b obtained from the reducing sugar calibration curve to obtain the concentration. (x). The resulting x value is then substituted into the formula for calculating the reducing sugar content of cassava plants with the addition of salicylic acid at several concentrations, presented in Table 2.

The observation results are based on Table 2. The reducing sugar content at a concentration of 0 ppm (control) was 19.376. The increase in reducing sugar content occurred from 80 ppm, namely 18,576 to 21,996 at a salicylic acid concentration of 100 ppm. Then at a concentration of 120 ppm it became 26,016 and followed by 32,276 at a concentration of 140 ppm. These results show that there is an influence on the administration of salicylic acid, where the higher the concentration of salicylic acid given, the higher the reducing content found in the cassava plant. The increase in reducing sugars comes from starch and sucrose due to carbohydrate conversion. Some carbohydrates are also used for respiration and form other compounds, this causes reducing sugar levels to increase but carbohydrates according to [15].

This is in line with the results of Nurcahyani's research [16] which shows that there is an increase in reducing sugar content along with increasing fusaric acid concentration with a concentration of 120 ppm having the highest reducing

sugar content, namely reaching 6.82%. This can occur due to the effect of fusaric acid on cassava plantlets. An increase in reducing sugar content at each concentration is related to the presence of impact resistance. As well as the existence of induced resistance mechanisms where there is activation of natural resistance in plants such as cell lignification (liginification), production of phytoalexins, and an increase in reducing sugar content.

Table 2 Reducing sugar content in cassava plants induced with salicylic acid for 30 days

Salicylic Acid Concentration (ppm)	Reducing sugar content (%)
0	19,716±933[a]
80	18,576±100[a]
100	21,996±100[b]
120	26,016±888[c]
140	32,276±037[b]

Note: Numbers followed by different letters indicate there are significant differences between treatments.

According to research by Baharudin *et al*., [17] stated that the higher the reducing sugar content, the higher the resulting color index (darker), this is because reducing sugar can affect the color produced after analysis. So, conversely, if the reducing sugar content is low, the resulting color index will be bright. Changes in reducing sugar levels are influenced by several factors, one of which is the heating process. Heating when testing sugar levels causes the chemical structure to change. This heating causes the glycosidic bonds to break and the non-reducing sugar (sucrose) breaks down into reducing sugars such as glucose and fructose. Apart from that, the reducing sugar content is also influenced by the administration of salicylic acid.

According to research by Al-kayyis [10] stated that the Somogyi-Nelson method has higher sensitivity and accuracy than other methods, so it is recommended to test the reducing sugar content with this method. In the Semogyi-Nelson method, there is a reaction between specific alkaline Cu (Cu2+) and reducing sugar to form Cu+ which has formed a brick red precipitate, then when arsenomolybdate solution is added, the reaction will dissolve and form a complex that changes color to greenish blue. In this spectrophotometric technique, data analysis of a number of mono- and disaccharides is used which only describes the presence of reducing levels.

6. Conclusion

The results of the research showed that there was a missing protein band, namely a band at a molecular weight of 115 kDa (treatment with a salicylic acid concentration of 140 ppm), and the appearance of a new band, namely at a molecular weight of 85 kDa (treatment with a salicylic acid concentration of 100 ppm). Reducing sugar content in cassava plants (*Manihot esculenta* Crantz) increases as the concentration of salicylic acid administered increases.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors have no conflicts of interest.

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