Resistance validation of several chili peppers to anthracnose (*Colletrothicum acutatum*) isolate Sukabumi

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Abstract. Anthracnose *Colletrothicum acutatum* is the most dominant and virulent disease that attacks hot pepper pants and can reduce yields. One of the ways to control anthracnose can use resistant varieties. The research objective was to ensure the resistance of the hot pepper lines to C. *acutatum* isolate from Sukabumi. Anthracnose isolates used *C. acutatum* from Sukabumi with a DNA band measuring 490 bp. The method used to test the resistance to anthracnose on hot pepper was by inoculating 5 ul of conidial suspension with a density of 5.0 x 10^5 as much as one injection per fruit. PR protein analysis was performed on anthracnose infected and healthy fruit. The results showed that: (1). Based on the morphology of the isolates, the top looks are white and gray, and beige, white, peach for the colony color is shown below. (2). *C. acutatum* isolates from Sukabumi were identified by PCR method with DNA bands measuring 490 bp. (3). The results of validation of resistance to anthracnose *C. acutatum* with Sukabumi isolates obtained resistant lines: 2A (R-4), 4A (R-15), 4B (R-9), 6 (R-12), 7 (-13), 8 (R-14) with a lesion diameter less than 5 mm. (4). The absorbance value of peroxidase enzyme and protein content of anthracnose-resistant fruit was higher than that of susceptible fruit. (5). The results of the analysis of the protein band pattern profile on the PR-protein results showed that in certain molecular weight range thin and thick gene expressions were seen with molecular weights ranging between 37 kDa.

1. Introduction

National chili production in 2017 was 1,206,266 tons with a harvest area of 142,547 ha and an average productivity of 8.46 tons/ha [1]. The potential yield of local red chilies can reach 12-20 tons / ha and the potential yield of hybrid red chilies can reach 20-30 tons/ha [2]. One of the reasons for the low productivity of chili cultivation is an important disease, namely anthracnose as a biotic factor. *Colletotrichum acutatum* species is the first anthracnose species reported to be virulent and the most dominant in Indonesia compared to *C. gloeosporioides* and *C. capsici* [3, 4]. The disease caused by the fungus *Colletotrichum* sp. attacks fruit, both newly formed and ripe, causing considerable losses. Anthracnose disease can cause chili yield losses of 25% - 90% [5, 6, 7, 8]. This fungal infection in chilies is characterized by brownish-yellow rot followed by wet rot which sometimes appears black soot, severe attacks can cause the whole fruit dries,

while the seeds can cause sprouts to fall [9, 10]. A fungal infection that causes anthracnose disease in chilies shows symptoms that are influenced by plant genetics and fruit physiology [11]

Controlling pathogens that cause anthracnose disease is not easy because these pathogens are often found in farmers' fields. Varieties that are resistant to the disease can sometimes be broken by the emergence of a new breed of a pathogen. Even plants cannot survive and produce well due to the many attacks that come at once. Anthracnose control using fungicides is not effective, especially in root attack [12]. One of the effective measures to control the aforementioned diseases is to use resistant varieties. Until now, anthracnose control still focuses on the use of synthetic fungicides. According to [13] the use of chemical pesticides at the farm level is high. Control that only relies on synthetic fungicides continuously and is supported by unwise use can cause various negative impacts on the environment and human health as well as high costs, causing high pesticide residues in chilies [14, 15].

Along with the stipulation of chili as a national strategic commodity, efforts are needed to support the availability of chili supply. One solution that can be done is the use of varieties that are resistant to pests and plant diseases, one of which is anthracnose. The first step that can be taken to overcome the problem of anthracnose is to test the resistance of varieties to the disease. This can also be a contribution in the field of plant breeding to reduce the use of pesticides by farmers.

Based on the results of previous studies, six lines were selected for resistance to the anthracnose *Colletotrichum acutatum* which needed to be reselected or validated to reconfirm their resistance level with the established resistance standards. Endurance test validation is a re-confirmation that the resistance of red chilies to anthracnose disease that has been tested previously fulfills the requirements for its intended use, namely as a donor source for assembly of chili varieties which is influenced by the level of plant resistance tested.

2. Materials and methods

The research was conducted at the Indonesia Vegetable Research Institute with an altitude of 1250 meters above sea level. The research was carried out in the laboratory from January to December 2019.

2.1. Rejuvenation and propagation of Colletotricum acutatum isolate Sukabumi isolates

The source of the inoculum of the fungus came from a pure culture of the *C. acutatum* fungus. Isolates from Sukabumi were identified by the PCR method and a DNA band measuring 490 bp in 2018. Fungi colonies isolated and purified were rejuvenated before being used in resistance experiments. Selected *C. acutatum* fungi from Sukabumi isolate were transferred to a petri dish containing PDA media then incubated at room temperature for 10 days. To ascertain the intended *Colletotricum* strain, it is carried out checking for spores under a microscope. If the fungal colonies match the characteristics of the intended pathogen, it is transferred to PDA media again. Isolation and conidia checks were repeated to ensure the purity of the isolates.

2.2. Validation of resistance test for chili fruit lines

Tests were carried out on selected lines resistant to anthracnose *C. acutatum* isolate Sukabumi. Endurance test validation was carried out by five personnel as a test and used a randomized block design. Tests were carried out by inoculating the fruit of anthracnose with *C. acutatum* isolate from Sukabumi. The third chili fruit yields were tested against red chilies. 6 genotypes. Each treatment unit (box) consists of five (5) chilies taken from 5 sample plants. After 7 days on PDA the fungi culture was watered with aquadest and conidia was taken from the dish. The inoculum density was adjusted to 5.0 x 105 conidia/ml using a hemocytometer.

The test procedure is carried out with: (1) The chilies were cleaned using tissue paper. (2) The test storage box was cleaned and sterilized with 70% alcohol. (3) Distilled water put into a ± 50 ml box (under a filter). (4) Chili arranged into boxes on top of the filter. Each chili is inoculated with 5 ul spores of conidia suspension (1 chili consists of one point). (5) Boxes were closed and labeled and incubated at room temperature. (6) Observations were made 3, 4, 5, 6 and 7 days after incubation (HSI).

2.3. Testing of peroxidase enzyme activity (modified method of Loebenstein and Lindsev)
One gram of chili leaves was put into a mortar and cooled at 4ºC for 30 minutes. The sample was crushed and added 0.2 M phosphate buffer pH 6.0 with a ratio (1:3). The leaf saps were filtered with gauze and centrifuged at 10,000 rpm at 4 0C for 10 minutes. A 0.05 pyrogallol solution was prepared (must be fresh). 5 ml of the aforementioned Pyrogalol solution was added with 6,250 ml of phosphate buffer 0.2 M pH 6.0 then diluted to 50 ml. 5 ml of the aforementioned solution plus 0.5 ml of 1% hydrogen peroxide solution. The solution mentioned above is taken 1 ml then put into a spectrophotometer cuvette and read at a wavelength of 420 nm. 18 ul supernatant was inserted into the cuvette above and homogenized then read the absorbance again. One minute change in absorbance was recorded for each sample.

2.4. Determination of dissolved enzyme protein levels was carried out based on the modified Lowry method
A total of 0.1 ml of enzyme was added with 0.9 ml of distilled water, reacted with 5 ml of reagent C and stirred evenly then left for 10 minutes at room temperature. After that, quickly added 0.5 ml of reagent D and stirred thoroughly for 30 minutes at room temperature. For control used 1 ml aquadest, then treated the same as the sample. The absorption was measured using a wavelength UV-Vis spectrophotometer at 750 n. To determine the dissolved protein content, the standard Bovin Serum Albumin (BSA) curve was used.

2.5. PR protein analysis
Protein content analysis was performed on fruit samples infected with anthracnose and healthy plants (negative control). The plant protein was extracted using modified Sun's method. Protein extraction was analyzed using the electrophoresis method at SDS-PAGE 10%. The steps taken for protein analysis were protein extraction, gel preparation and isolate horesis. The separating gel solution was prepared using 8.0 ml of distilled water (H_2O); 5 ml Tris-HCl 1.5 M pH 8.8; 0.2 ml SDS 10%; 6.7 ml Acrylamide; 0.1 ml ammonium persulfate (APS) 10%, and 0.010 ml TEMED. Stacking gel solution was prepared using 6.1 ml distilled water (H_2O), 0.5 ml Tris HCl, 0.5 M pH 6.8; 0.1 ml SDS 10%; 0.05 ml 10% APS and 0.010 ml TEMED. Each sample of 10 ul was inserted into the sunur. Protein detection in the gel was done by staining the coomassie blue overnight and shaking it using a shaker. The dye solution consists of methanol 45.5%, 45.5% H2O, 9% acetic acid, and 0.09% coomassie blue R 250. Gel storage was carried out by soaking the gel in a 7% acetic acid solution. Observations were made visually on the thickness of the protein band pattern.

Variable being measured consists of observation of *C. acutatum* isolate Sukabumi as a result of rejuvenation and propagation; 2) validation of resistance test for lines/varieties of chilies; 3) changes in absorbance of peroxidase enzyme activity; 4) protein content; and 5) PR protein. The collected data were analyzed statistically, and the difference between the two means was tested using Duncan's multiple distance test (UJBD) at a 5% confidence interval.

Observation of the width of the lesions was carried out by measuring the diameter of the lesions at 3 to 7 days after inoculation. The resistance category uses criteria [16] which divides the resistance level into five classes, chili genotype is categorized as resistant if it has a lesion diameter smaller than 5 mm (Table 1).

Table 1. Resistance level.

Number	Diameter lesion (mm)	Resistance level:
1	Immune	Diameter = 0
2	Resistance	0 < Diameter ≤ 5
3	Mildly resistance	5 < Diameter ≤ 10
4	Some what suseptible	10 < Diameter ≤ 20
5	Suseptible	> Diameter 20

3. Results and discussion

3.1. Rejuvenation and propagation of C. acutatum isolate Sukabumi

Isolate *C acutatum* from Sukabumi, the results of research in 2018, which was isolated and identified under a microscope, was verified by testing using the PCR method and amplification obtained by a DNA band measuring 490 bp. Based on the BLAST results in the database, the homology is 100%, the Query Cover is 100% with *Colletothricum acutatum*. The isolates above are rejuvenated and reproduced, obtained from the results of microscopic observations, distinguished from the color of the isolates based on the colony color, which shows quite a variety of color variations, namely for the colonies appear on white and gray, and beige, white, peach for the colony color below (figure 1). According to [17], colonies of *C. acutatum* were white at first and then became pink or orange in color and [18], described colonies of *C. acutatum* as white at the beginning of development then turning orange and gray.

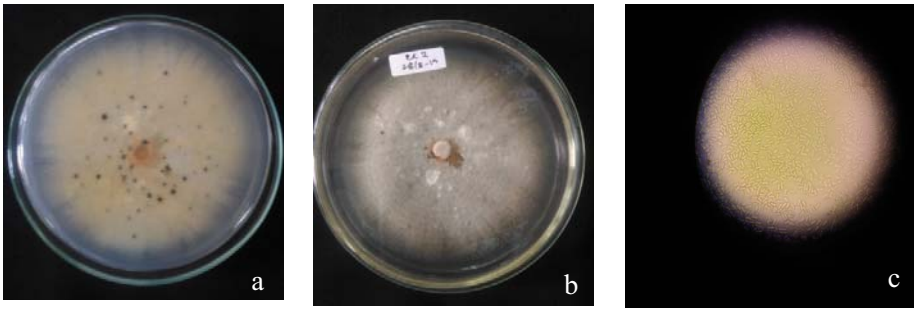


Figure 1. *C. acutatum* isolate from Sukabumi. (a) The rejuvenation of Sukabumi isolates can be seen from below, (b) The rejuvenation of Sukabumi isolates can be seen from above, (c) Conidia of *C. acutatum*

3.2. Identification of isolates using the PCR (Polymerase Chain Reaction) method

The results of isolation and single spore multiplication of Sukabumi isolates were used for testing the PCR method. The use of PCR techniques with ITS primers is because the sequence has a broad spectrum to identify various fungi. The DNA amplification process used Calnt2 and ITS4 primers. According to [19], Calnt2 and ITS4 are universal primers that can amplify and identify *C. acutatum* fungi. As many as 5 isolates of *C. acutatum* from West Java could be amplified with a DNA band measuring 490 bp (figure 2). Based on the BLAST results in the database, the homology is 100%, the Query Cover is 100% with *C. acutatum*.

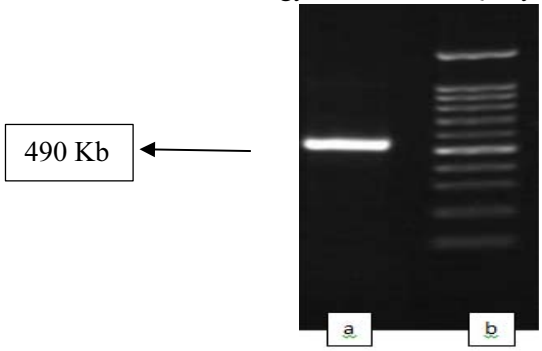


Figure 2. Identification results of anthracnose isolates from Sukabumi with PCR method. (a) *C. acutaum* isolate from Sukabumi; (b) (M) = leader DNA marker.

3.3. Validation of resistance test of lines/varieties of chilies

The validation test for the diameter of the lesio was carried out by five personnel on the six selected chili lines as a result of the resistance test selection of 28 line/varieties. Observation 3-5 days after inoculation, the lesion diameter varied and was not significantly different between chili lines (table 2).

Table 2. Validation of the average diameter of the anthracnose lesions of *C. acutatum* Sukabumi isolates and the level of resistance in the red chili fruit lines/varieties.

No.	Line	Lesion width (Days after inoculation)					Level of Resistance
		3	4	5	6	7	
1.	2A (R-4)	0.21 a	1.08 a	2.34 a	3.50 a	4.00 a	VR
2.	4A (R-15)	0.21 a	0.67 a	1.67 a	2.02 ab	2.25 ab	VR
3.	4B (R-9)	0.27 a	1.01 a	1.55 a	2.87 ab	3.88 ab	VR
4.	6 (R-12)	0.22 a	0.72 a	1.01 a	1.26 b	1.34 b	VR
5.	7 (R-13)	0.39 a	0.87 a	0.95 a	1.56 ab	2.78 ab	VR
6.	8 (R-14)	0.14 a	0.79 a	1.04 a	1.57 ab	2.08 ab	VR

Note: VR = Very resistant

At 6-7 days after inoculation, there was a difference in the diameter of the lesions between lines. The smallest diameter of the lesion was in line 6 (R-12) and the widest inline 2A (R-4). The overall of all the lines that were validated, the diameter of the lesion was still below 4 mm with resistance levels including resistance: 2A (R-4), 4A (R-15), 4B (R-19), 6 (R-12), 7 (R-13), and 8 (R-14) (figure 3).

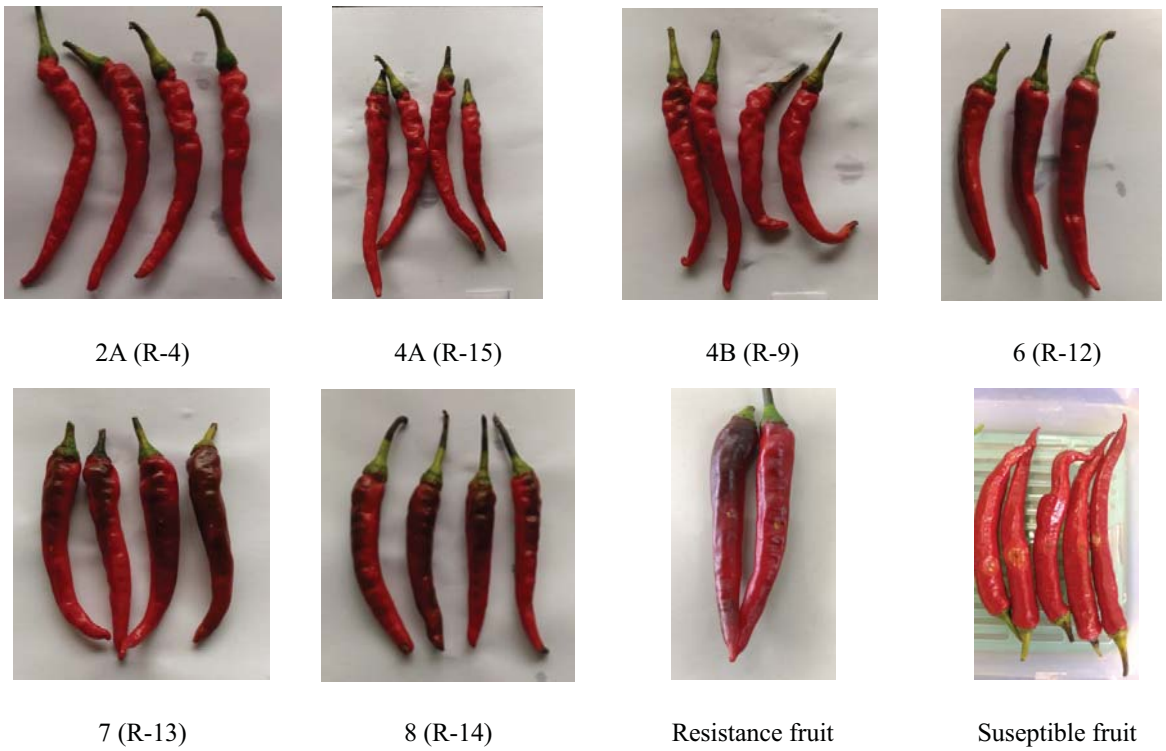


Figure 3. Results of the chili resistance test against *C. acutatum* Sukabumi isolates

3.4. Peroxidase enzymes

Peroxidase activity was measured at a wavelength of 420 nm at 0 minutes and 30 minutes after incubation. It can be seen that at 0 minutes in healthy controls the absorbance value of the peroxidase enzyme in the lines/varieties was lower than the fruit samples infected with the anthracnose *Colletotrichum acutatum* Sukabumi isolate. It can be seen that the absorbance value ranges from 0.019 to 0.046. This is because the fruit is not infected with anthracnose and is in normal conditions. In contrast to the fruit infected with anthracnose, the absorbance value was higher than that of healthy controls in the same line. The peroxidase enzyme was different between lines and higher than the sensitive control. In the 30th minute measurement, there was an increase in the absorbance value compared to the first minute for each line. The highest absorbance values were seen in line 6 (R-12) and the lowest in 8 (R-14) and there was a decrease in the absorbance value from the first minutes to 30 minutes (figure 4 and 5). Several studies on disease resistance studies suggest that peroxidase enzyme activity is associated with resistance and can be used as a marker for the selection of disease resistance [20-21].

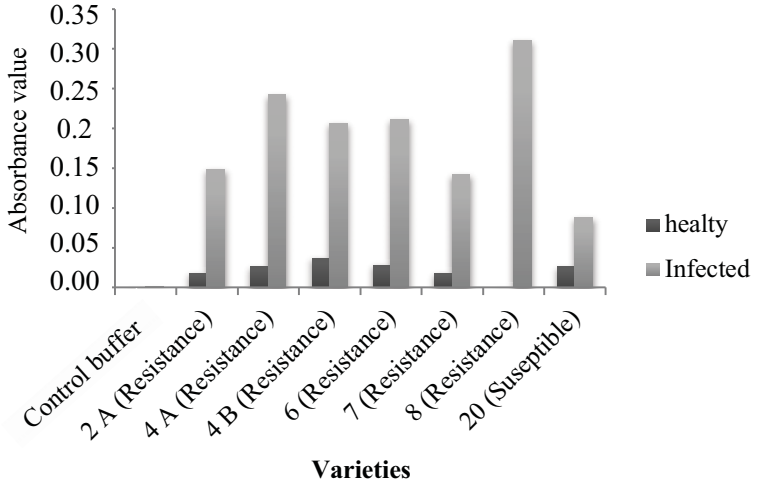


Figure 4. The results of the analysis of peroxidase enzymes are measured at wavelengths 420 nm at first minute.

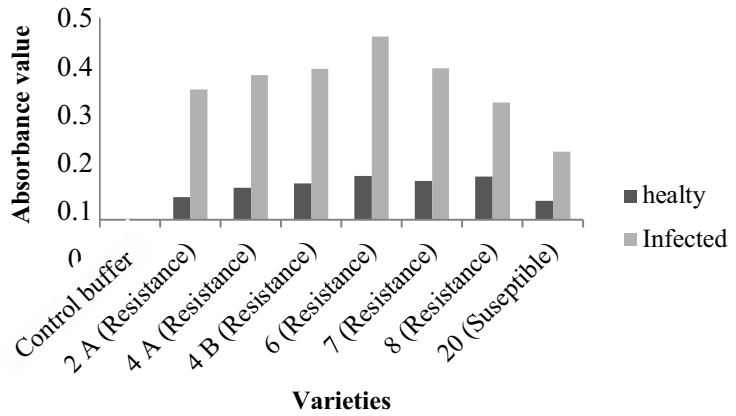


Figure 5. The analysis of peroxidase enzymes was measured at wavelengths 420 nm at 30 min.

3. 5. Dissolved protein content

Lesion on chilies infected with anthracnose isolates Sukabumi caused changes in protein levels (figure 6 and 7). It can be seen that in healthy controls the absorbance value of protein levels in the lines/varieties was lower than the fruit samples infected with the anthracnose *Colletotrichum acutatum* Sukabumi isolate. It can be seen that the absorbance value ranges from 0.001 to 0.078. This is because the fruit is not infected with anthracnose and is in normal conditions. In contrast to the fruit infected with anthracnose, the absorbance value was higher than that of healthy controls in the same line. Protein content looked different between lines and higher than sensitive controls. At the 30th minute measurement, there was an increase in the absorbance value compared to healthy controls in each line. The highest absorbance value was seen in line 4B (R-9), 6 (R-12) and the lowest was 2A (R-4). From the data above, it can be seen that the infection of chilies with anthracnose inoculum can change the protein activity higher, which indicates that the increase in protein activity is a form of functional resistance of genes from chili lines/varieties.

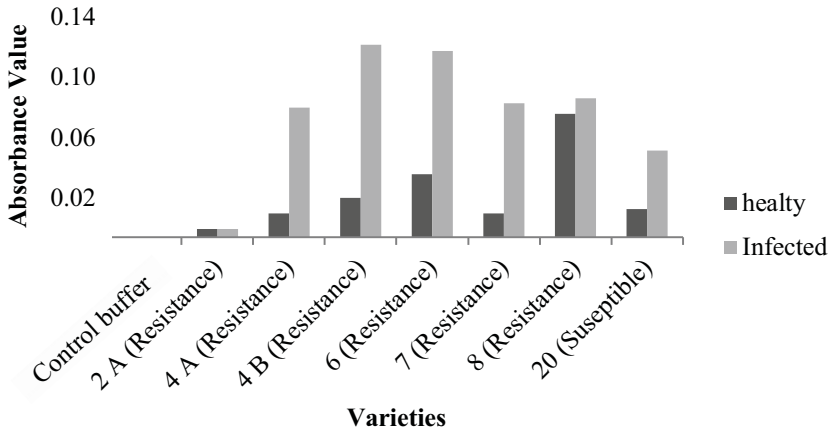


Figure 6. The results of the analysis of dissolved protein content are measured at wavelength of 750 nm.

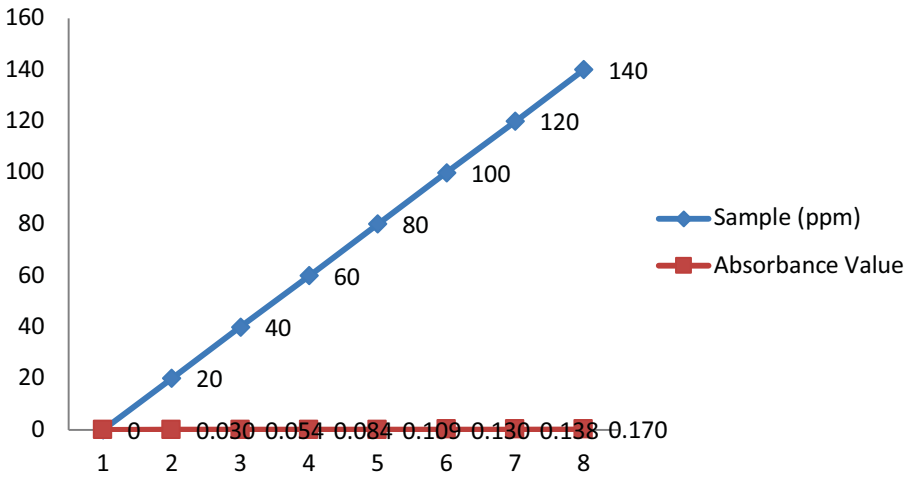
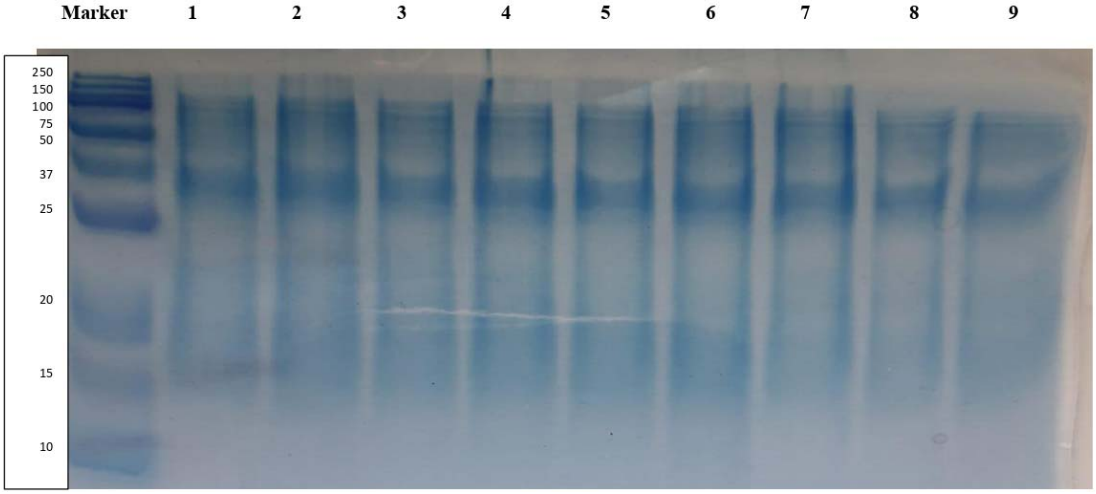


Figure 7. Standard curve of protein content (Bovin Serum Albumin).

3.6. PR (pathogenesis-related protein

Protein analysis in this study was carried out by separating proteins using the 2-dimensional SDS-PAGE electrophoresis method (figure 8). This separation is carried out to see if there are differences in the protein banding pattern of resistant, sensitive and healthy plants. The results of electrophoresis on polyacrylamide gel with a marker (M) with standard protein molecular weight (BM) between 10 kDa - 250 kDa (Marker Bio-Rad Precision plus dual color (10 - 250 kD). Results of profile analysis of protein band patterns on PR-Protein results show that a certain molecular weight range shows very thin and very thick gene expression The results of the separation of proteins from these samples show the presence of several protein bands with molecular weight (BM) ranging from 37 kDa Electrophoresis is a process in which protein/enzyme molecules have electricity moves through an electric field. The results of electrophoresis will be obtained by separated protein bands based on their molecular weight. The thickness of the ribbons formed from the protein bands indicates the content or number of proteins that have the same molecular weight in This is in line with the principle of movement of charged molecules, namely ber molecules charges can move freely under the influence of an electric field, molecules with the same charge and size will accumulate in the same or adjacent zones or bands [22]. Protein separation by electrophoresis method aims to see whether there are differences in protein profiles in resistant, sensitive and healthy samples.



Description: (1). 4A (R-15), (2). 4B (R-9), (3). K.4. A (Healthy Control), (4). 2A (R-4), (5). 22 (HK) (Sensitive Control), (6). 7 (R-13), (7). 8 (R-14), (8), K.7 (Healthy control), (9). 6 (R-12).

Figure 8. The results of protein PR (pathogenesis-related) testing in the six selected lines.

The results of research conducted by [23-27] showed that infection with pathogens can cause PR protein expression with different molecular weights due to anthracnose infection. PR protein was shown to be thicker, especially in plants that were resistant to anthracnose compared to sensitive plants. According to [28, 29] in measuring the total protein of resistant lines, the appearance of PR 1 protein tended to be thicker than the sensitive and control lines. This is probably because these proteins are proteins associated with the formation of cell walls.

4. Conclusions

Based on the morphology of the isolates, the top looks are white and gray, and beige, white, peach for the colony color is shown below. *C. acutatum* isolate from Sukabumi was identified by PCR method with DNA bands measuring 490 bp. The results of validation of resistance to anthracnose *C. acutatum* with Sukabumi isolates obtained resistant lines: 2A (R-4), 4A (R-15), 4B (R-9), 6 (R-12), 7 (-13), 8 (R-14) with a lesion

diameter less than 5 mm. The absorbance value of peroxidase enzyme and protein content of anthracnose-resistant fruit was higher than that of suceptible fruit. The results of the analysis of the protein band pattern profile on the PR-protein results showed that in certain molecular weight range thin and thick gene expressions were seen with molecular weights ranging between 37 kDa.

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