Virulence of five anthracnose Colletotricum acutatum isolates from West Java against the resistance of hot pepper

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Abstract. The most dominant disease attacked hot pepper plantations is anthracnose (Colletotrichum acutatum). This disease can attack fruit, causing substantial losses in quality and quantity of 20% - 90%. The purpose of this research is to select lines or varieties of hot pepper that are potentially resistant to Colletotrichum acutatum. This research was carried out at the Indonesia Vegetable Research Institute from January to December 2018. Samples of anthracnose-infected plants were taken from several locations in West Java. Identification of C. acutatum isolates was done by PCR method. The results showed that: (1) The intensity of anthracnose disease is different in five districts (Bandung, Garut, Tasikmalaya, Ciamis and Sukabumi) ranging 50% -73%. The high intensity of anthracnose symptoms depends on cropping patterns, used of varieties, age and plant conditions. (2) The morphology of the isolate the top view white and gray, and beige, white, peach for the colony the bottom. (3) C. acutatum isolates from West Java were identified by PCR, DNA band measuring 490 bp. (4) The isolates from Sukabumi were the most virulent. (5) Inoculation of 5 isolates from West Java on hot pepper were not obtained resistant lines / varieties.

1. Introduction
National hot pepper production in 2017 was 1,153,155 tons with a harvest area of 167,600 ha and an average productivity of 6,887 tons / ha, while hot pepper production was 1,206,266 tons, harvested area was 142,547 and average productivity was 8.46 tons / ha [1]. The potential yield of local hot pepper can reach 12-20 tons / ha and the potential yield of hybrid hot pepper can reach 20-30 tons / ha [2].

The low productivity of hot pepper is influenced by biotic and abiotic factors. One of the most important biotic factors in hot pepper plants is disease. Suryaningsih et al [3] stated that the most dominant disease attacking hot pepper cultivation is anthracnose (Colletotrichum sp.). Several species that cause anthracnose in Indonesia are Colletotrichum acutatum, C. gloeosporioides and C. capsici. The species C. acutatum was the first reported and most dominant species in Indonesia and was more virulent than C. gloeosporioides and C. capsici [4,5]. Five cultivars of national hot pepper and 10 cultivars of IPB collection, none of them were resistant to C. acutatum [6]. According to Kirana et al [7], genotype AVPP 0207 and Perisai are included in the anthracnose-resistant chili genotype. Sutoyo and Gniffke [8] reported that PP 0537-7558 is a chili genotype that has multiresistant resistance to anthracnose C. capsici, curly yellow virus and Phytophthora spp.
Anthracnose can reduce yield by 25-75% [9-11]. Disease caused by the fungus *Colletotrichum* sp. Combining newly formed and ripe fruits, causing considerable losses. Anthracnose can also reduce the quality of hot pepper, including decreased levels of phenol from 16 to 69%, capsaicin levels from 20 to 60%, and oleoresin levels from 17% to 55% [11]. This fungal infection in hot pepper is characterized by rotting yellow-brown fruit followed by wet rot which sometimes appears black soot, severe attacks can cause the whole fruit to dry out, while the seeds can cause sprouts to fall [12,13]. This pathogen attacks hot pepper plants in the highlands and lowlands [14].

Controlling pathogens that cause anthracnose disease is not easy because these pathogens are often found together 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. Until now, anthracnose control still focuses on the use of synthetic fungicides. According to Ameriana [15] using 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 hot pepper [16,17]. One of the ways to control anthracnose is to use resistant varieties of hot pepper.

Research activities for selection of resistance to anthracnose in the 20 proposed genotypes are part of a series of studies where each stage of the research in a series of activities as a whole has specific objectives, which consist of, 1) obtaining effectiveness in increasing the genetic resistance of hot pepper to disease, 2) obtain hot pepper lines that are resistant to anthracnose and can be used as a parent source of anthracnose resistance genes that can be combined with other parents with other superior properties with the ultimate goal to contribute to efforts to increase farmers' income through reducing the risk of loss of yield due to infection with the fungal anthracnose *Colletotrichum acutatum*.

This study aims to select hot pepper lines / varieties that are potentially resistant to anthracnose (*C. acutatum*).

### 2. Materials and methods

The research was carried out in two stages, namely a survey in the field (West Java) and the laboratory of the Vegetable Crops Research Institute with a height of 1250 meters above sea level, from January to December 2018.

#### 2.1. Hot pepper fruit sampling

The method of sampling hot pepper in the field was carried out by survey methods and stratified multi-stage sampling and purposive sampling. Each district is selected from three to five districts. Each district was selected from one to three hot pepper planting villages, from each village one to three hot pepper planting plots were selected. The hot pepper fruits taken are red and cayenne peppers that have anthracnose symptoms, then the chilies are taken to the laboratory for testing.

#### 2.2. Fungus isolation

Infected hot pepper from the field were disinfected using 70% alcohol. The boundary between healthy and diseased tissue is cut and put in 1% Clorox solution for 1 minute, then dried on sterile filter paper. After drying the pieces of fruit are placed on the media Potato Dextrose Agar (PDA), then incubated at 25 °C for 10 days. *C. acutatum* isolates were purified using a single spore method.

#### 2.3. Morphology of *C. acutatum*

Microscopic observations of the morphology of the growing fungal colonies were carried out 10 days after isolation (HSI). Observations were made on the color of the colonies above and below, and the colony diameter on PDA media. Observations were made using a slide preparation and a microscope, then documented with a digital camera.
2.4. Pathogenicity test of C. acutatum
The pathogenicity test used Kencana variety. The hot peppers were cleaned using tissue paper. The test storage box was cleaned and sterilized with 70% alcohol. Put the aquadest in the box ± 50 ml (under the filter). Hot pepper arranged into boxes on top of the filter. Each hot pepper was inoculated with 5 ul spores of conidia suspension (1 hot pepper consists of one point). Boxes were closed and labeled than incubated at room temperature. Observations were made 3, 4, 5, 6 and 7 days after incubation (HSI). The hot pepper that had been treated were incubated in a closed container under humid conditions and temperature of 25 °C. The variables observed were the average lesion diameter and virulence measured at 7 days after inoculation. Pathogenicity score from 0-3 (Table 1).

| Score | Lesion Measure | Virulence Level |
|-------|----------------|-----------------|
| 0     | There is no    | Non-virulent    |
| 1     | < 3 mm         | Low             |
| 2     | 3 – 5 mm       | Moderate        |
| 3     | > 5 mm         | High            |

2.5. DNA extraction
DNA of five Colletotrichum acutatum isolates was extracted by taking hyphae then placed in a centrifuge bottle and suspended with 100 ul PrepMan Ultra reagent. The sample was vortex for 30 seconds then placed in a hot block at temperature 95 0C - 100 0C for 10 minutes then centrifuged at 10,000 rpm for 2 minutes and the pellets were taken as DNA extraction and used for further analysis.

2.6. DNA amplification of C. acutatum
DNA amplification was performed on a Thermo Cycle PCR machine. Amplification was performed using specific primers for C.acutatum, namely forward primer Calnt2 (5’GGGGAAGCCTCTCGCGG-3’) and reverse primer ITS4 (5’-TCCTCCGCTTATTGATATGC-3) with the target size of the amplification result is 490 bp. DNA amplification was performed with a total volume of 25 µL consisting of 2 µL DNA, 2.5 µL 10x buffer and Mg2+, 0.5 µL Dntp 10 mM, 1 µL each primer 0.2 µL Taq DNA (5 units / µL), and 17.8 µL H2O. The amplification conditions were divided into several stages, which were 94°C pre-saturation for 1 minute, 45°C primer for 1 minute, 72°C DNA synthesis for 2 minutes, especially for the last cycle plus the synthesis stage for 10 minutes, then the cycle ended with a temperature of 4°C.

2.7. DNA electrophoresis
The amplified product was analyzed with 1% agarose gel (0.5x Tris -Borate EDTA / TBE). Electrophoresis at 50 volts for 50 minutes and then the agarose gel was incubated in a dye containing ethidium bromide (1%) for 15 minutes, then washed with H2O for 10 minutes. The results of the electrophoresis were visualized with an ultraviolet transilluminator. The DNA bands formed in the electrophoretic results were documented with a digital camera. BLAST testing for databases was carried out at ICBB (Indonesian Center for Biodiversity and Biotechnology in Bogor).

2.8. Resistance test of 20 hot pepper lines / varieties
The treatments tested were as many as 20 lines / varieties hot pepper: Tanjung-2-S (CB, OP), Inata Agrihort (CB, Hybrid), Tanjung-2-P (CB, OP), Pilar (Sensitive control) (CB, Hybrid), PP 0537-7558 (Hold Control) (CB, OP), Perisai (Control Hold), (CB, OP), MS 006-1 (CB, OP), Chiko (CB, OP), Lingga (CB, OP), MS-005-1 (CB, OP), 2016/2 C. 816 (CK, OP), Tampar Ungu (CK, OP), Kencana (CB, OP), MS-007-5 (CK, OP), MS-007-3 (CK, OP), H-1 (CR, Hybrid), H-4 (CK, Hybrid), HK (CK, Hybrid), MS-001-5 (CK, OP). The study used a randomized block design (RBD) which was repeated 3 times. Each treatment unit (box) consists of five (5) chilies taken from 5 sample plants. The source of the inoculum of the fungus came from a pure culture of C. acutatum, an isolate from Sukabumi, which...
was identified by the PCR method and a DNA band measuring 490 bp was grown on PDA media, after 7 days on PDA the fungi culture was doused with aquadest and conidia was taken from a dish. The inoculum density was adjusted to 5.0 x 10^5 conidia / ml.

Testing Procedure: The hot peppers were cleaned using tissue paper. The test storage box was cleaned and sterilized with 70% alcohol. Put the aquadest in the box ± 50 ml (under the filter). Hot pepper arranged into boxes on top of the filter. Each hot pepper was inoculated with 5 ul spores of conidia suspension (1 chili consists of one point). Boxes were closed and labeled than incubated at room temperature. Observations were made 3, 4, 5, 6 and 7 days after incubation (DAI).

Observations were made on:
1). General conditions and incidence of anthracnose symptoms.
2). Morphological observations of C. acutatum isolates
3). Virulence of five C. acutaum anthracnose isolates
4). Endurance test of 20 lines / varieties of hot pepper.

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 category of resistance uses criteria which divides the level of resistance into five classes, namely immune, resistant, mildly resistant, quite sensitive and sensitive (Table 2), hot pepper genotype is categorized as resistant if it has a lesion diameter smaller than 5 mm.

| Number | Diameter lesion (mm) | Resistance level: |
|--------|---------------------|------------------|
| 1      | Immune              | Diameter = 0     |
| 2      | Resistance          | 0 < Diameter ≤ 5 |
| 3      | Mildly resistance   | 5 < Diameter ≤ 10|
| 4      | Somewhat susceptible| 10 < Diameter ≤ 20|
| 5      | Susceptible         | > Diameter 20    |

Data collected were statistically analyzed, and the difference between the two averages was tested using Duncan's multiple range test (UJBD) at a 5% confidence interval.

3. Results and discussion

3.1. Field: General conditions of planting and incidence of anthracnose symptoms in hot pepper plants in west java

Observation and sampling locations for anthracnose infected fruit in Bandung Regency are in Pengalengan sub-district in four locations (Desa Sukamanah, Pulasari, Sukaluyu and Mekar). Garut Regency as many as four Subdistricts namely Banyuresmi District two locations (Sukaraja-1 and Sukaraja-2), Bayongbong sub-district one location (Ciniisti Village), Cisurupan District two locations (Tambakbaya Village and Balewangi District), and Cilawu District one location (Cilawu village). Tasikmalaya Regency five Districts, namely Kadipaten District, one location (Mekarsari Village), Rajapolah District one location (Manggungsari Village), Kawalu District one location (Talagasari Village). There are three districts of Ciamis, namely Kawali District (Kawali Mukti Village), Panjalu District (Mapara Village), Sukamantri District one location (Cibeurem Village). Sukabumi Regency are three Subdistricts namely Sukalarang Subdistrict two locations (Titisa Desa -1 and Titisan -2), Caringin District one location (Caringin Village), Cisaat Sub-district one location (Cisaat Village) (Table 3).

The general condition of planting in the field generally varies between locations. The observed land area is between 300 m^2 - 6300 m^2, hybrid planted varieties, OP (Open Polineted) consisting of large chili, curly chili and cayenne pepper, plant age between 90 - 365 days after planting. The cropping patterns are intercropped with other plants such as petsay, tomatoes, Chinese cabbage, eggplants, red beans and cabbage which are planted between hot pepper and on the edge of the beds. Symptoms of anthracnose incidents in plants different between locations ranging from 50% - 73%.
Table 3. General conditions for planting and the incidence of anthracnose symptoms in the hot pepper plant west java

| No. | District/sub-district | Village | Land area (m²) | Varieties | Age of Plants (Day) | Intercropping | Anthracnose Incidents |
|-----|-----------------------|---------|----------------|-----------|---------------------|---------------|-----------------------|
| 1.  | District Bandung      |         |                |           |                     |               |                       |
|     | Sub-district          |         |                |           |                     |               |                       |
|     | Pangalengan           |         |                |           |                     |               |                       |
|     | Sukumanah             | 350     | Cayenne        | 180       | Petcay              | Anthracnose   | 40%                   |
|     | Pulasari              | 300     | Cayenne        | 180       | Monoculture         | Anthracnose   | 60%                   |
|     | Sukaluyu              | 700     | Curly          | 120       | Tomatoes, Chinese cabbage | Anthracnose | 60%                   |
|     | Marga Mekar           | 560     | Curly          | 365       | Tomatoes, Chinese cabbage | Anthracnose | 60%                   |
| 2.  | District Garut        |         |                |           |                     |               |                       |
|     | Sub-district          |         |                |           |                     |               |                       |
|     | a. Banyuresmi         |         |                |           |                     |               |                       |
|     | b. Banyuresmi         | 3000    | Curly          | 240       | Monoculture         | Anthracnose   | 80%                   |
|     | c. Bayongbong         | 6300    | Curly          | 40        | Ginger              | Anthracnose   | 50%                   |
|     | d. Cisurupan          | 2500    | Curly          | 70        | Monoculture         | Anthracnose   | 80%                   |
|     | e. Cisurupan          | 3000    | Curly          | 360       | Monoculture         | Anthracnose   | 30%                   |
|     | f. Cilawu             | 700     | Curly          | 180       | Monoculture         | Anthracnose   | 80%                   |
|     | g. Cilawu             | 2000    | Curly          | 70        | Monoculture         | Anthracnose   | 60%                   |
| 3.  | District Tasikmalaya  |         |                |           |                     |               |                       |
|     | Sub-district          |         |                |           |                     |               |                       |
|     | a. Kadipaten          | 3000    | Large Tanjung  | 110       | Monoculture         | Anthracnose   | 2%                    |
|     | b. Rajapolah          | 1500    | Curly          | 145       | Eggplant            | Anthracnose   | 10%                   |
|     | c. Kawalu             | 2000    | Large Tanjung  | 175       | Monoculture         | Anthracnose   | 10%                   |
| 4.  | District Ciamis       |         |                |           |                     |               |                       |
|     | Sub-district          |         |                |           |                     |               |                       |
|     | a. Kawali             | 1000    | Large Tanjung  | >120      | Monoculture         | Anthracnose   | 80%                   |
|     | b. Panjalu            | 750     | Curly          | 120       | Monoculture         | Anthracnose   | 95%                   |
|     | c. Sukamantri         | 2000    | Hybrid Penam   | 120       | Tomato and Red beans | Anthracnose   | 5%                    |
|     |                     | 2000    | Curly Kastilo  | 150       | Monoculture         | Anthracnose   | 25%                   |
|     |                     | 3000    | Cayenne        | 150       | Monoculture         | Anthracnose   | 90%                   |
| 5.  | District Sukabumi     |         |                |           |                     |               |                       |
|     | Sub-district          |         |                |           |                     |               |                       |
|     | a. Sukalarang         | 3000    | Curly var. Rimbun OR | 90   | Monoculture         | Anthracnose   | 5%                    |
|     | b. Caringin           | 1000    | Curly Phonix   | 150       | Intercropping cabbage | Anthracnose   | 60%                   |
|     | c. Cisaat             | 1500    | Cayenne        | 150       | Monoculture         | Anthracnose   | 80%                   |
|     |                     | 1000    | Curly          | 180       | Monoculture         | Anthracnose   | 70%                   |

3.2. Laboratory

3.2.1. Number of anthracnose isolates and Virulence Isolates. The number of anthracnose isolates from 5 districts in West Java (Bandung, Garut, Tasikmalaya, Ciamis and Sukabumi) was obtained 77 samples of anthracnose isolates. Virulence test was performed on cayenne pepper and curly hot pepper obtained by virulent isolates based on the widest lesion width and observations under a microscope, 4 isolates from Pangalengan, 6 isolates from Garut, 3 isolates from Tasikmalaya, 3 isolates from Ciamis and 3 isolates from Sukabumi. The above isolates were taken one per district based on the widest diameter of virulence test results and used to test the resistance of 20 lines / varieties of hot pepper. The widest lesion diameter appears of the Pangalengan from Marga Mekar, Garut isolate from Tambakbayan, Tasikmalaya isolate from Mekarsari, Ciamis isolate from Kawali Mukti, and Sukabumi isolate from Titisan. The most virulent isolates of the five isolates mentioned above are isolates from Sukabumi (Table 4)
Table 4. Number and virulence isolat of anthracnose

| No. | District/Sub-district | Village | Number of Anthracnose Isolat Samples | Diameter Lesion (mm) | Virulence Rate |
|-----|-----------------------|---------|------------------------------------|---------------------|----------------|
| 1.  | District Bandung      | Sukamanah | 5                                  | 2.11                | Low            |
|     | (Sub-district. Pangalengan) | Pulasari   | 5                                  | 1.26                | Low            |
|     |                       | Sukaluyu  | 6                                  | 2.28                | Low            |
|     |                       | Marga Mekar | 6                                | 3.49                | Medium         |
| 2.  | District Garut        | Sukaraja  | 4                                  | 5.22                | High           |
|     | a. Sub-district Banyuresmi 1 | Sukaraja | 8                                  | 3.11                | Medium         |
|     | b. Sub-district Banyuresmi 2 | Suwaraja | 4                                  | 3.41                | Medium         |
|     | c. Sub-district Bayongbong | Cinisti   | 2                                  | 6.44                | High           |
|     | d. Sub-district Cisurupan | Tambakaya | 3                                  | 4.71                | Medium         |
|     | e. Sub-district Cisurupan | Balewangi | 5                                  | 5.94                | High           |
|     | f. Sub-district Cilawu | Cilawu    | 4                                  | 5.94                | High           |
| 3.  | District Tasikmalaya  | Mekarsari | 3                                  | 5.94                | High           |
|     | a. Sub-district Kadipaten | Mekarsari | 2                                  | 5.97                | High           |
|     | b. Sub-district Rajapolah | Manggungsari | 2                               | 4.01                | Medium         |
|     | c. Sub-district Kawal | Talagasari | 1                                  | 4.01                | Medium         |
| 4.  | District Ciamis       | Kawali Muki | 8                                | 2.50                | Low            |
|     | a. Sub-district Kawali | Mapara    | 9                                  | 1.76                | Low            |
|     | b. Sub-district Sukamantri | Cibeureum | 1                                | 1.19                | Low            |
| 5.  | District Sukabumi     | Tekisan   | 2                                  | 12.11               | High           |
|     | a. Sub-district Sukalarang | Tekisan | 2                                  | 8.05                | High           |
|     | b. Sub-district Caringin | Caringin | 2                                  | 10.67               | High           |

**Total** 19 Location 77

3.2.2. **Morphology of C. acutatum Anthracnose Isolat.** The diversity of the five isolates of *C. acutatum* from West Java that had been isolated single spore and identified under a microscope was distinguished from the colors of the colony looks quite varied namely the colonies appear to be white and gray, the color of the colonies bottom view to be creamy, white and peach. According to [18, 19], the *C. acutatum* colony is white at first and then becomes pink or orange, describes the *C. acutatum* colony which is white at the beginning of its development and then becomes orange and gray.

3.2.3. **Molecular identification of C. acutatum.** The verification of the five isolates from single spore (Pangalengan, Garut, Tasikmalaya, Ciamis and Sukabumi) was verified by testing using the PCR method. The DNA amplification process using the specific primer *C. acutatum* Calnt2 / ITS4 in the five isolates tested showed a 490 bp band size (Figure 1). According to [20, 21], Calnt2 and ITS4 are universal primers which can amplify and identify *C. acutatum* fungi. Based on the results of BLAST in the database obtained homology of 100%, 100% Query Cover with *Colletothricum acutatum*. 
Note: 1 = Tasikmalaya, 2 = Sukabumi, 3 = Ciamis, 4 = Pangalengan, 5 = Garut, (M)= leader DNA marker, (K-) = positive control

Figure 1. Results of identification of anthracnose isolates from West Java with PCR testing technique

3.2.4. Screening resistance of hot pepper lines/varieties

Screening of 20 lines / varieties of hot pepper using five selected isolates from West Java seemed to provide varied responses to the diameter of lesion in hot pepper. The widest and most virulent lesion diameter was seen in Sukabumi isolates followed by Pangalengan. As many as 20 strains / varieties of red chili tested with the five isolates mentioned above were not obtained which were resistant, this was due to lesion diameter of more than 5 mm (Figure 2).

Note: CB = large hot pepper, CK = curly hot pepper, CR = cayenne pepper

Figure 2. Diameter of 20 lines / varieties of lesions which were inoculated using 5 isolates anthracnose C. acutatum from Java West

The same lines / varieties gave different responses to the five isolates from West Java, although morphologically the isolates cannot be distinguished. It seems that the resistance of hot pepper to anthracnose C. acutatum is strongly influenced by the lines / varieties and virulence from which the isolate was used. The origin of isolates, virulence and varieties greatly influenced the ability to infect hot pepper [22]. Anthracnose disease can attack on fruit that have been red or the fruit that still green [23,24]. According to Tenaya [25], C. capsici attacks on fruit that have been red are more severe than
those that are still green, this is caused by fruit that is red already containing glucose, sucrose and fructose which is considered to have an important role as a trigger for the development of *C. capsici* compared to fruit that are still green which only contain sucrose and fructose. Resistance to a disease such as anthracnose is controlled by resistance genes that are expressed in plant morphology that support the mechanism of resistance to the disease.

4. Conclusion
The incidence of anthracnose disease is different the crops of five districts (Bandung, Garut, Tasikmalaya, Ciamis and Sukabumi) ranging between 50% - 73%. The high incidence of anthracnose symptoms depends on cropping patterns, varieties, plant age, and plant conditions. Based on the morphology of the isolates the top view white and gray, the bottom new and beige, white, peach for the colony color. *C. acutatum* isolates from 5 districts in West Java were identified by the DNA band PCR method measuring 490 bp. Isolates from Sukabumi were the most virulent compared to 4 other isolates (Pangalengan, Garut, Tasikmalaya and Ciamis). The results of inoculation of 5 isolates from West Java on hot pepper fruits did not obtain resistant strains / varieties

Acknowledgement
Main author is thankful and appreciation goes to Dr. Redy Gaswanto, SP, MP as a PI activity who has given advise, motivation, and taught about discipline

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