Production of Pectinase Enzymes by *Colletotrichum acutatum* Simmonds. Causing Anthracnose in Red Chilli (*Capsicum annuum*, L.)

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Abstract. Almost all pathogenic fungi produce pectinase enzymes used to degrade host cell wall during pathogenesis. The mode of action of *C. acutatum* (Ca) is no different from other *Colletotrichum* species. The fungi produce cutinase and pectinase enzymes to weaken the host defenses and help the further infection. The success of pathogenesis by *C. acutatum* determines the occurrence of anthracnose disease. During pathogenesis, other than cutinase, pectinase is an enzyme that plays a vital role in cell wall degradation of the host. This paper discusses the ability of *C. acutatum* to produce pectinase enzymes. The fungus of six isolates was the *C. acutatum* from Java whose pathogenicity was the highest at each sampling location (Malang, Temanggung, Kulonprogo, Brebes, Garut, and Pandeglang). The results showed that all *C. acutatum* isolates produced pectinase. There was a difference in the ability to macerate enzymes even though enzyme quality test showed no difference.

1. Introduction

In Indonesia, red chili is one among those cultivated-horticulture plants because of its wide range adaptability to various altitudes and climates. The plant, however, is susceptible to anthracnose when cultivated in a humid area with rainfalls of 600-1250 mm p.a. So far, Java is known as the main center of red chili production. Anthracnose of *C. Acutatum*, however, limits the red chili production in Java. The disease has also been reported worldwide in tropical and subtropical countries. Some environmental factors, such as warm air temperature and humidity, influence the severity and dispersal of the plant pathogen. Wet and humid leaves, for example, have a direct correlation with the level of severity. Apart from those factors, rainfall intensity and duration, leaf humidity and sunlight also take a significant role in pathogenicity as they are correlated with germination, and penetration to the host tissues [1].

At a favorable environment, the anthracnose symptoms appear as a concave spot or circling on the mature red chili fruit, leading to severe fruit rot. The spot starts from the formation of black-spot on the concentric circle, which under a microscope it becomes evident as acervulus with hairy-setae to trap conidia of the pathogen. The pathogen commonly forms microscopic-sclerotia in the host tissues to survive during the unfavorable condition. Pathogen infests all parts of the plant including leaves and stem, as small concave brown-greyish spots with darkening edges.
The C. acutatum mode of action is similar to those fungi caused another anthracnose [2]. The Colletotrichum produces cutinase, before weakening the host defense system, to be able to infest it completely [3]. It also produces pectinase to degrade pectin polymers and hydrolizing the cuticles. Apart from those two enzymes, the fungus produces a toxic compound called colletotrichins to block the growth of germinated host and predict to cause anthracnose [4]. The more virulent Colletotrichum produces more pectinase than no virulent one. In culture, the pectinase enzyme is produced optimally at 10 days old and undergoes a lag phase afterward [5].

On the appropriate substrate, i.e., in the presence of pectin on its growing medium, the enzyme has secreted the pathogen to split the cell wall [5]. Maceration of the particular enzyme would be therefore crucial in the testing of the pectinase production [6]. Other variables might be the dry weight mycelium [5], the clear zone [5], and the nondegraded pectin [7, 5].

This study aimed to discuss the ability of six C. acutatum isolates from Java in producing pectinase enzymes. The isolates have been recognized to have the highest pathogenicity at each sampling location (Malang, Temanggung, Kulonprogo, Brebes, Garut, and Pandeglang).

2. Methods

Six isolates of C. acutatum were collected from Malang, Temanggung, Kulonprogo, Brebes, Garut, and Pandeglang were along with this study as they were the most virulent fungi.

Test of the macerating enzyme was done in three different growth media (1) ammonium oxalate medium consisted of 0.5% glucose, 0.389% ammonium oxalate, 0.1% potassium dihydrogen, and 0.05% magnesium sulphate, (2) modified Richard’s medium: 1% potassium nitrate, 0.5% potassium dihydrogen phosphate, 1.0% magnesium sulphate, 2.5% glucose and trace ferric chloride, and (3) glucose-asparagine medium: 0.5% glucose, 0.5% asparagine, 0.05% magnesium sulphate and 0.1% potassium dihydrogen phosphate.

All growing media were added with 1% pectin. Each medium consisting of a volume of 25 ml was put into 150-ml-Erlenmeyer flasks and set to pH of 5.5 before sterilization in the autoclave (15 minutes, 2 atm). Inocula of each isolate with 8-mm-diameter were cultured in each growing medium, incubated at room temperature no longer than 10 days. Observations were done on day 2, 4, 6, 8, and 10. The mycelium was sieved through Whatman paper (no 41), dried in the oven (60°C for two consecutive days), and weighed. The filtrate was centrifuged (4000 rpm, 20 minutes), and the supernatant was taken, classified as an enzyme, then used in the test of the macerating enzyme. Ten pieces of potato (8 mm diameter, 0.5 mm thick) were placed in a sterile petri dish, and each petri dish was added with 10 ml filtrate of the fungal culture. The disc on the potato was observed every 6 hours using a glass rod. The activity of the enzyme was calculated as disc maceration each hour. The control dish was filled with the medium in the absence of inoculum.

Test of pectinase quality was done on the CDA growing media which were formulated as 2 g sodium nitrate, 1 g potassium nitrate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.3 g ferrous sulfate, 30 g sucrose, and 20 g agar. In this research, 1% pectin substituted the sucrose. An 8-mm-diameter inoculum with five replicates was placed in the center of the growing medium. After 10 days, a clear area on the growing medium was observed, and the diameter was measured. The clear area indicated pectinase production by the C. acutatum.

Test of pectinase quantity was done in a CD liquid medium which formulated as 2 g sodium nitrate, 1 g potassium nitrate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.3 ferrous sulfates, and 30 g sucrose. Again, 1% pectin substituted the sucrose. The 8-mm-diameter C. acutatum colony was cultured in the CD medium (5 replicates), incubated in room temperature for 10 days. At the end of the culturing period, the culture was sieved using a Whatman paper no.1. The filtrate was observed using an Ultraviolet-visible (UV-vis) spectrophotometer (575 nm wavelength), and pure pectin as a standard solution.
3. Results

On day 2 of the incubation period, the inoculum of Ca-2 and Ca-4 isolates did not produce protopectinase (macerating enzyme). The macerating enzyme activity was observed on day 4 (Table 1). The Ca-3, however, showed macerating enzyme activity faster than any other isolates.

| Isolate | Incubation period | Macerating enzymes | Macerating time (h) |
|---------|-------------------|--------------------|---------------------|
|         |                   |                    | 1  | 2  | 3  | 4  | 5  |
| Ca-1    | 2                 |                    | 48 | 48 | 48 | 48 | 48 |
| Ca-1    | 4                 |                    | 48 | 48 | 48 | 48 | 48 |
| Ca-1    | 6                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-1    | 8                 | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-1    | 10                | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-2    | 2                 | No macerating      | 48 | 48 | 48 | 48 | 48 |
| Ca-2    | 4                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-2    | 6                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-2    | 8                 | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-2    | 10                | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-3    | 2                 | No macerating      | 48 | 48 | 48 | 48 | 48 |
| Ca-3    | 4                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-3    | 6                 | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-3    | 8                 | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-3    | 10                | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-4    | 2                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-4    | 4                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-4    | 6                 | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-4    | 8                 | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-4    | 10                | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-5    | 2                 | No macerating      | 48 | 48 | 48 | 48 | 48 |
| Ca-5    | 4                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-5    | 6                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-5    | 8                 | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-5    | 10                | No macerating      | 24 | 24 | 24 | 24 | 24 |
| Ca-6    | 2                 | No macerating      | 48 | 48 | 48 | 48 | 48 |
| Ca-6    | 4                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-6    | 6                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-6    | 8                 | No macerating      | 36 | 36 | 36 | 36 | 36 |
| Ca-6    | 10                | No macerating      | 36 | 36 | 36 | 36 | 36 |

The least dry weight of Ca isolates cultured in CDB+ pectin was noted from Ca-1 and Ca-2 incubated for five days. However, when a more extended incubation period was provided, their dry weight was similar to others. Both Ca-3 and Ca-6 isolates, observed at all incubation period (day 5, 10 and 15) showed a non-significant different dry weight indicating both isolates (on day 5 and 15), produced a similar amount of mass (Table 2).
The smallest clear area was recorded from Ca-6 isolate grown in the CDA+ pectin medium, whereas the largest was from Ca-4. The clear area of Ca-1, Ca-2, Ca-3, and Ca-5, however, did not differ from Ca-4 or Ca-6 suggesting all isolates were able to utilize pectin as a source of carbon for their growth and development. Such amount of pectin left in the substrate was also utilized by the Ca-2, Ca-4, Ca-5, and Ca-6 isolates for carbon source on their growth on day 5, 10, and 15. Two isolates, Ca-1 required 15 days for utilizing pectin but only 10 days for Ca-3 isolate. The clear zone produced by the isolates is presented in Table 3.

Table 3. The average number of the clear zone in the cultural medium of Ca isolates

| Isolates | The average number of the clear zone (mm) |
|----------|------------------------------------------|
| Ca-1     | 3,606±0,326ab                            |
| Ca-2     | 3,400±0,675ab                            |
| Ca-3     | 3,130±0,414ab                            |
| Ca-4     | 4,000±0,306a                             |
| Ca-5     | 3,354±0,832ab                            |
| Ca-6     | 2,924±0,491b                             |
4. Discussion

During the pathogenesis period, almost all isolates produced pectinase enzymes to degrade the host cell wall. Besides, the cutinase enzyme was also taken a significant role in degrading the host cell wall. The ability of *C. acutatum* to start pathogenicity determines the presence of anthracnose. All isolates were able to produce a pectinase enzyme. These data are in line with the general phenomenon of this fungi. The macerating enzyme (protopectinase) of all isolates, however, varied greatly depending on their incubation period (Table 1). The longer the incubation period, the higher the ability in macerating protopectinase. These results coincide with a similar finding that more virulent isolate produces a more macerating enzyme, which is parallel with the incubation period [7].

Table 2 indicates the dry weight of all isolates. The fungi were able to use pectin as a carbon source for their growth and development. They required Nitrogen and Carbon to grow well, as reflected by the clear zone area on the medium. The zone reflects the pectin degradation in the medium by pectinase enzyme produced by the fungi. Current data were in line with a published study [7] reporting that the pectinase was produced maximally after 10-day incubation. The presence of pectinase indicates its role in pathogenicity level. The dry weight increased parallel with its incubation times. The more virulent the fungus, the more dry weight of the mycelium is produced [6].

The formation of the clear area reflects the isolate ability to degrade pectin as a source of carbon for their growth and development. Current data showed that the Ca-4 isolate had the highest ability in degrading pectin (Table 3). The Ca-1, Ca-2, Ca-3, and Ca-5 isolates showed a similar ability in degrading pectin. The clear area on the medium indicates the isolates were virulent because they were able to degrade pectin, a vital compound of plant cells wall. Pathogenicity succeeds when the pathogen produces pectinase enzyme, and degrades the pectin compound of the plant cell wall.

5. Conclusion

The fungal ability to produce pectinase is strongly related to its pathogenicity. The Ca-4 isolate showed the best performance of degrading pectin in the culture, although, on day 2, it did not secrete the macerating enzyme. On day 4, however, this isolate demonstrated the fastest macerating enzyme.

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