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Inhibitory response of *Pectobacterium carotovorum subsp. carotovorum*(Jones) Dye to Betel Leaf and Areca Nut Extract as a Potential Organic Bactericide

Respon Penghambatan Pertumbuhan Bakteri *Pectobacterium carotovorum subsp. carotovorum*(Jones) terhadap Ekstrak Daun Sirih dan Pinang sebagai Bakterisida Organik Potensial

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ABSTRACT

Soft root caused by bacterial *P. carotovorum subsp carotovorum*, is a detrimental disease to horticultural plants including carrot. The disease is commonly controlled using a chemical technique. Given the negative impacts of the technique, it is important to search environmentally friendly techniques, such as plant extracts. This research aimed to determine the growth response of P.c.c bacteria to the extracts of betel leaves (EDS) and Areca nut (EBP) in different concentrations. A pathogenecity test, gram-coloring test, and growing on a selective media was conducted, while the inhibition testing was carried out In Vitro. The experiments were undertaken by growing bacterial inoculum on an agarose-wells medium added with the plant extracts. The data of inhibitory zone diameter was then analyzed using ANOVA followed by Duncan's test. The results showed that the symptoms of red and rod-shaped bacteria appeared 4 days after infection. The bacteria grew uniformly on the CVD medium at 37^0C without the formation of basin. The result of in vitro showed that the treatment of 10% EDS and 5 and 10% EBP concentration had a strong ability to inhibit bacterial growth with the average inhibition zone diameters of 32.33 mm, 28.67 mm, and 33 .67 mm.
Key words: Antibacterial extracts, plant-based pesticide, soft rot

ABSTRAK

Busuk lunak yang disebabkan oleh bakteri *P. carotovorum* subsp *carotovorum* (Jones) Dye (P.c.c), merupakan penyakit yang sangat merugikan tanaman hortikultura termasuk tanaman wortel. Pengendalian penyakit ini umumnya dilakukan secara kimiawi. Mengingat dampak negatifnya, maka perlu dicari teknik yang lebih ramah lingkungan, yakni pemanfaatan bahan nabati. Penelitian ini bertujuan untuk mengetahui respon pertumbuhan bakteri P.c.c terhadap ekstrak daun sirih (EDS) dan buah pinang (EBP) dengan konsentrasi yang berbeda. Penelitian ini diawali dengan uji patogenesitas, uji pewarnaan gram dan pertumbuhan di medium CVD. Sedangkan pengujian penghambatan dilakukan secara in-vitro. Metode yang digunakan adalah sumuran agar, dimana inokulum bakteri ditumbuhkan bersamaan dengan media agar, kemudian ekstrak nabati

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ditambahkan ke dalam media melalui sumuran. Pengamatan dilakukan dengan mengukur diameter zona hambat di sekitar sumuran agar. Data selanjutnya dianalisis ragam dan uji lanjut DMRT. Hasil analisis menunjukkan adanya gejala 4 HSI, bakteri berwarna merah dan berbentuk batang. Sedangkan pada medium CVD, bakteri tumbuh seragam pada suhu 37^0 C, walaupun tidak membentuk cekungan pada media agar. Hasil uji *in vitro* menunjukkan bahwa perlakuan EDS 10% dan EBS 5% dan 10% memiliki kemampuan untuk menghambat bakteri, dengan rata-rata diameter zona peghambatan sebesar 32.33 mm, 28.67 mm, dan33.67 mm.
Kata kunci : Busuk lunak, ekstrak Nabati anti bakteri, pestisida berbahan dasar tanaman

INTRODUCTION

Soft rot is a disease that harms horticultural crops such as carrots, mustard greens, tomatoes, potatoes both in the field, in storage and in transportation as a post-harvest disease. The disease is caused by a group of pathogenic bacteria *P. carotovorum* subsp. carotovorum (Jones) Dye (P.c.c). In Indonesia, the disease has also been identified in orchids using the PCR method and amplifying *P. carotovorum*'s 16S rRNA gene (Joko et al, 2011). The most common symptom in plants is soft rot in the infected part, producing an unpleasant odor. Bacterial infections cause changes both physically and chemically in plant products and even in the seeds to be planted (Schaad & Jones, 2001).

The common control is mechanically burning the residues of infected plants, using biocontrol agents such as *Bacillus subtilis* and P*seudomonas fluorescens* (Javandira et al, 2013), whereas chemically in the field using synthetic pesticides (Supriadi et al, 2002). The use of synthetic pesticides that are unwise besides being not economical, often has a negative impact on the environment, human health, environmental damage, and the influence of pathogen resistance. Therefore, efforts should be made to control plant pathogens, by applying the use of natural and environmentally friendly control materials, one of which is by using plant-based pesticides (Rustama et al, 2005). Some plants have been known to be able to act as antifungal, antibacterial and plant-based insecticides, including betel plant (*Piper betle* L.), and areca nut (*Areca catechu* L.).

Betel leaf contains essential oils that inhibit microbial growth such as phenolic (Row & Ho, 2009), sterols (Chakraborty & Shah, 2011) and are antibacterial such as safrol and cavibetol acetate (Arambewela et al , 2005), while

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according to (Friedman et al, 2002) Antimicrobial components of betel leaf are carvacrol, eugenol, cavibetol, and eugenol isomers. The extracts known being able to inhibit the growth of microbes such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Listeria monocytogenes* and *staphylococcus aureus* (Suliantari et al, 2008). Areca nut seeds contain flavonoids and alkaloid content which are useful as antibacterial (Wang & Lee, 1996). Areca extract has a good inhibitory ability against the growth of *Streptococcus mutans* bacteria (Yulianeri, 2006). However, due to the content of the two substances from different plant extracts, so does its ability to inhibit the growth of certain bacteria. Therefore, research needs to be carried out aimed at analysing the response of the bacterium *P. carotovorum* subsp *carotovorum* to the vegetable extracts of betel leaves and betel nuts at different concentrations

MATERIAL AND METHODS

The activities carried out in the laboratory included bacterial multiplication, pathogenicity test, gram staining test, and growth test in selective mediums. While the inhibitory test was carried out in vitro, including bacterial dilution, manufacture of vegetable pesticide extracts, and implementation of inhibitory test of bacterial growth on agar media. The bacteria used were pure Pcc bacterial isolates obtained from the Plant Bacteriology Sub-Laboratory, Plant Disease Laboratory of Gadjah Mada University, while betel leaf extract (EDS) was obtained from green betel leaf, and betel nut extract (EBP) was obtained from dried young areca nut. Gram-bacterial staining observations on bacterial gram staining included bacterial color and bacterial shape. Gram positive or gram negative bacterial groups were determined by identifying the color change at the end of the coloring process. The positive gram is purple, while the negative Gram is red (Waluyo, 2008).

Pathogenicity Test. The pathogenicity test was carried out by inoculating the P.c.c bacteria in the pathogen host, namely carrot bulbs and potato tubers from a 10-6 dilution suspension as much as 1 mL, so that bacterial pathogenicity can be seen which is characterized by the appearance of black spots and rot symptoms (Semangun, 2000). The growth test was performed in the Crystal Violet Pectate

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(CVP) Selective Medium. The test was carried out by taking 100 µL of P.c.c. bacterial culture in the NB medium. The CVP selective medium was spread with sterile spreader rods and then incubated for ± 24-48 hours at 370ºC, and observed. Bacterial growth was observed by looking at the degradation of the CVP medium due to the reaction of the pectinase enzyme in the form of a basin on the surface of the medium and the uniformity of bacterial colony and the suitainability of the growth environmental temperature (Schaad & Jones, 2001). In Vitro test was carried out in the Laboratory of In vitro treatment to measure the inhibition zone of 2 types of plant extracts at 2 levels of concentration and a one control of the growth of *P.c.c* bacterial colonies with 3 replications. So that there were 15 experimental units. The design used was a completely randomized design (CRD).

Method of Making Vegetable Pesticide Extract

Betel leaves used were young leaves of the green betel, whilst the young betel nuts were characterized by having solid flesh and there was no space between the flesh and skin. Betel leaves and betel nuts were then sliced and dried at room temperature for about one week, and then blended to produce flour. The flour was dissolved based on concentration needed. For example, a concentration of 5%: as much as 1 gram and 10% as much as 2 grams in 20 mL of distilled water and incubated for 24 hours at room temperature. The solution was filtered twice with Whatman filter paper No. 41, so that a clear filtrate was obtained to be further used in the treatment.

Bacterial dilution

Bacterial dilution was performed to get the best dilution that were used in the test. Culture used for dilution of 100 - 10^{-9} levels was the culture from sloping agar. The suspension used in the treatment was a suspension of 10^{-6}, with the following stages: a single colony of bacteria was prepared in order to tilt at 37^0C and incubated for 24 hours. The bacterial colony was diluted 10^{-1}-10^{-6} times by preparing test tubes containing 9 mL of distilled water each with a dilution form (10^{-1}; 10^{-2}; 10^{-3}; 10^{-4}; 10^{-5}; 10^{-6}) and arranged in a row. The suspension of the *P.c.c* bacteria culture was shaken with hand until it was evenly distributed, and then the culture was aseptically taken with a pipette of 1 mL and put in a 10^{-2} dilution blank and slowly shaken. Samples from the 10^{-1}

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dilution test tubes were taken as much as 1 mL, then transferred to the 10^{-2} dilution blank, then shaken following the previous step. The step three was repeated until the 10^{-6} dilution form made. Next, three petri dishes were prepared and the outer bottom edges were labeled 10^{-4}; 10^{-5}; 10^{-6}. Each sample (0.1 mL) from a 10^{-4}, 10^{-5}; 10^{-6} dilution bottle were aseptically transferred into the petri dish with their respective blanks. Spreaders used was sterilized by dipping them in 70% alcohol, then burned with Bunsen until the alcohol ran out. The suspension was spread on the 10^{-4}, 10^{-5}; 10^{-6} petri dishes aseptically using a spreader stem. Each petri dish was incubated at upside down position in an incubator for 24 hours, and then counted the number of colonies growing on the surface of the medium. The number of microorganisms per mL of culture was calculated at 24 hours by multiplying the colony count with the dilution factor and divided by the colony forming unit / mL (CFU / mL).

In-Vitro Test of Inhibitory Extracts Against Bacterial Growth

Determination of inhibition was based on Capuccino & Sherman (1983) and Prescott et al., (1990). Media nutrient agar (NA) of 10 mL was poured aseptically into a sterile petri dish and allowed to freeze as a base layer. As much as 5 mL of NA cold medium was heated at a temperature of 45^0-48^0C, and a 1 mL of bacterial culture was prepared. The NA medium and the 10^{-6} dilution of bacterial isolates were combined, and then poured over the base layer of the medium and spread evenly. Aluminum sprinkles were then placed on the medium as much as 2 points and vegetable bactericides and distilled water samples were labeled respectively. Each well was added with 200 µL of vegetable extract and 200 µl of distilled water as control, and then incubated at 37^0C for 24 hours. Furthermore, inhibition zone diameter (mm) was observed for 4 days (96 hours) after infection.

Data analysis

Data on the measurement of inhibition zone diameter were then analyzed using analysis of variance. If the analysis had a significant different, the analysis was then continued to Duncan Multiple Range Test (DMRT) of 5%.

RESULTS AND DISCUSSION

Propagation of Bacteria

Bacterial isolates are rejuvenated to get a single bacterial colony. A single

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colony is a colony that grows from one bacterial cell of a kind. The morphological characteristics of the bacterial colony *P.c.c.* are whitish yellow, shiny surface, slippery colony edge, slimy and wetness (Figure 1.a). The result of identification based on the gram staining method showed that bacteria was in the form of stem and red in color, which indicated the gram-negative group (1.b). Gram-negative bacteria are bacteria that do not maintain crystal violet dyes in the Gram staining method, and absorb dyes from safranin. This was due to the structure of Gram-negative bacterial cell walls, especially the peptidoglycan layer, which was thinner than the cell walls of Gram-positive bacteria. Whereas in the pathogenicity test it was found that bacterial isolates caused spoilage in carrot and potato tubers 4 days after isolation (HSI) (Figures 1.c and 1.d).

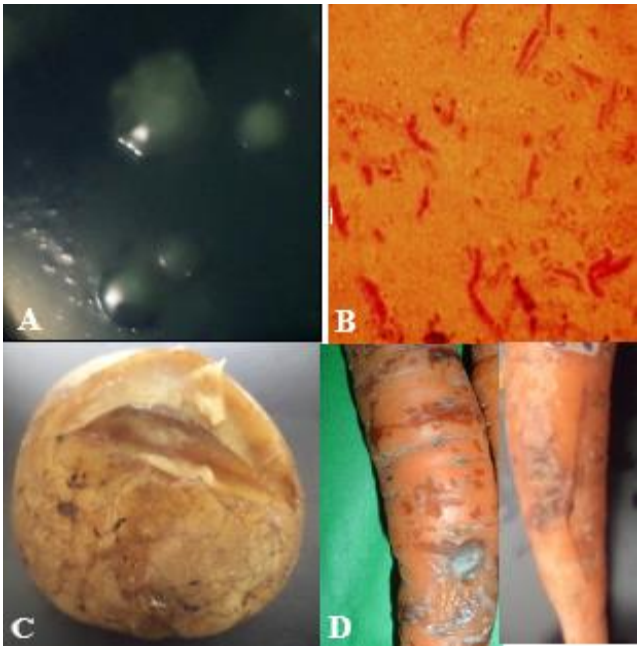

Figure 1. Bacterial culture a). The bacterial colony of P.c.c. with the characteristic whitish, shiny, slimy, slippery and wetness, b) Gram staining results; the form of stem bacteria and red (Mikrosoft light; magnification 40x), c) and d) pathogenicity test on potatoes and 4 HSI carrots; looks soft and slimy rot.

The results of testing on the CVP media did not show any changes on the surface of the medium in the form of a basin. This phenomenon was likely due to the influence of agar media density. However, there were other criteria in the

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identification and characterization of P.c.c on the CVP medium according to De Boer & Kelman (2001) namely biochemistry and physiology. Based on this test, it was noted that the bacterial colonies also grew uniformly on the CVP selective medium at temperature 37^0C in incubator, with the characteristics of a whitish yellow colony, with the edge of the colony being slippery, shiny, and slimy.

Inhibition of extracts against bacterial growth

The results of the EDS and EBP inhibition test (5% and 10%) on bacterial growth showed that the EDS and EBP extract significantly affected the diameter of the bacterial growth inhibition zone at 1% level (Table 1). The treatment of E (EBP 10%) was insignificantly different from treatment D (EBP 5%) and C (EDS 10%), but significantly different from the treatment B (EDS 5%) and A (Control).

Table 1. Mean Zones of P.c.bacteria Inhibiting Growth Zone after being treated with betel leaf extract and areca nut for 4 days on agar media

Treatment	Average and Notation	
A(Control)	0.00	a
B(EDS 5%)	25.00	b
C (EDS 10%)	32.33	bc
D (EBP 5%)	28.67	bc
E(EBP 10%)	33.67	c

Note: The numbers followed by the same letters are not significantly different in the 5% DMRT test

Inhibitory zone is an area around the well that is not found the growth of bacteria (Yulianeri, 2006; Ainurrochma et al, 2013). The antibacterial activity of betel leaf extract and betel nut in inhibiting the growth of P.c.c bacteria was known by measuring the diameter of the inhibitory zone and then reducing the diameter of the wells. The EBP has a compound of secondary metabolites such as tannin (15%) (Kristina & Syahid, 2007), and antibacterial alkaloids, while the inhibitory ability of EDS is related to the phenolic compounds contained in EDS extract that can be used as an antibacterial and antifungal from phenol derivatives namely cavikol in its

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antiseptic properties (Pelczar, M.J, 1988).

The result also showed that the presence of antibacterial activity was characterized by the formation of a zone of inhibition of bacterial growth (Figure 2). These observations indicated that an increase in the concentration of treatment of EDS (10%) will have a good effect on the diameter of the inhibition zone compared to a low concentration (5%). In contrast, the control showed no inhibitory ability which can be seen in the mean value of the inhibition zone. This was because water does not have organic compounds that was antibacterial.

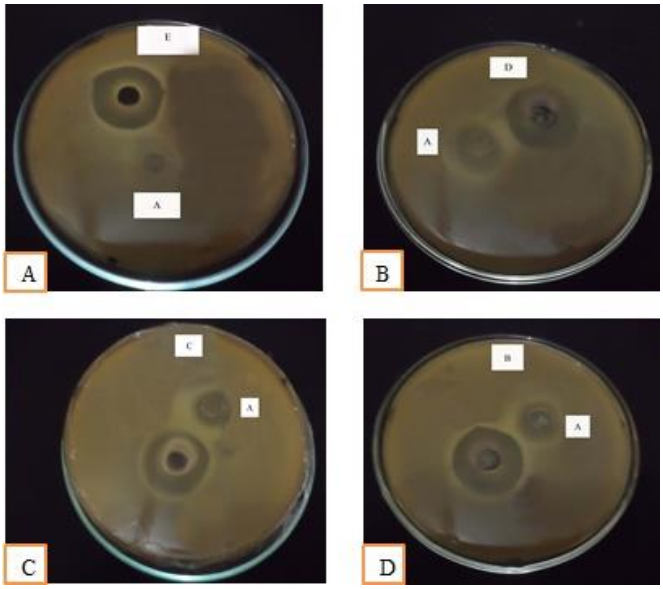

Figure 2. EDS and EBP antibacterial test results: a) EBP 10%, b) EBP 5 %, c) EDS 10 %, d) EDS 5%. Label A on petri disk is a control treatment (sterilize distilled water).

The mechanism of bacterial inhibition by secondary metabolite compounds produced by plant extracts was different, namely by damaging and inhibiting the formation of cell walls. It, therefore, will facilitate other compounds to interact with other cell constituent components of bacteria. The alkaloid compounds had the same activity in damaging the cell wall through the constituent components of peptidoglycan in bacterial cells. The red dragon fruit peel extract containing tannin was also known to have antibacterial properties against *S. Pyogenes* (Suhartati & Roziqin, 2017),

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while phenol works in killing microorganisms by denaturating cell proteins (Pelczar, M.J, 1988).

CONCLUSION

The EDS and EBP extract had ability to inhibit the growth of *P.c.c*. This inhibitory ability varied depending on the optimal concentration. The EDS was identified to have a high inhibitory ability at the concentration of 10%, whereas the EBP at the concentration of 5% was able to provide a high inhibition zone, with an average value of inhibition zone diameter for EDS 10%, EBP 5% and 10% was of 32.33 mm, 28.67 mm and 33.67 mm, respectively. This was suggested to do further study on the direct inhibitory ability of bacterial host plants.

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