Virulence and genetic diversity of Phytophthora isolates associated with cocoa pod rot

T Kuswinanti, Muhammad Junaid, Baharuddin and M Melina

Plant Pest and Disease Department, Faculty of Agriculture, Universitas Hasanuddin, Makassar, 90245, Indonesia.

E-mail: koeswinanti@yahoo.com

Abstract. Phytophthora palmivora is an important pathogen of cocoa in the world and it has a widest host-range. High adaptability to host plants and changes in environmental conditions forms a new evolutionary strain among P. palmivora populations. The purpose of this study was to analyze the virulence and genetic diversity of the isolates associated with cocoa pod rot symptoms. The isolates (infected cocoa pods) collected from Bone, Bulukumba and Pinrang regencies were characterize morphologically and molecularly. For morphological identification, Phytophthora black pod disease was determined by hyphal growth on juice V8 agar medium and for genetical and virulent identification, pathogen was tested with primer pairs of ITS4 and ITS5 and the diversity was then analyzed with the BOXA1R primer. The Hyphae of isolates were found to have stellate pattern, ovoid sporangium, smooth globose oogonium and oospores. Of 21 isolates, 14 isolates were obtained from Bone, 6 isolates from Bulukumba and 3 isolates from Pinrang respectively and testing virulence of the isolates after reinoculated onto the pod layer shown that seventeen (17) isolates expressed a much higher virulent level and only four (4) isolates performed moderately level. DNA fragments of isolates were amplified at 900 bp and the highest genetic diversity among isolates were 67% level.

1. Introduction

Cocoa is an important commodity of rural economy in South Sulawesi. It contributes to around 85% of the total national production. The South Sulawesi Plantation Service reported in 2015, that cocoa production amounted to 140,317 tons, with the largest producers in Luwu Regency totaling 27,640 tons, followed by North Luwu with 22,160 tons, and Bone District with 14,308 tons. However, cocoa production achieved tends to decline from year to year. In 2017, production only reached to 112,381 kg/ha, when compared to 2016 amounting to 115,326 kg/ha [1]. However, there are constraints of growing a better cocoa nowadays and one of the most is pest and disease infestation [2,3]. Regarding major cocoa disease, Phytophthora palmivora species is the most important cocoa pathogen [3,4]. P. palmivora alone causes pod rot symptoms and reduces productivity and quality of cocoa beans. The pathogen has a broad host range and causes global yield loss of up to 20-30% [5]. In the wet season, yield losses due to pod rot can reach at 100% [6, 7].

The pathogen has a wider host-range and is a very high adaptive species causing genetic drift based on morphological and genetic variations among other pathogens. The genetic drift forming a new strain is also supported with the presence of seedling clone variation and agronomic aspect. In South Sulawesi, analysis of P. palmivora genetic diversity regarding virulent diversity has not been widely explored specially in different main cocoa area. Once we have virulent diversity of pathogen based on
location, the control of Phytophthora pod rot disease will be applied based on specific location. Therefore, the paper will explain the variation of morphological and genetic *P. palmivora* isolates obtained from main cocoa areas in South Sulawesi.

2. Materials and methods

Sample collection was carried out from cocoa plantations in South Sulawesi Province in three districts namely Bone, Bulukumba and Pinrang. Laboratory testing was conducted at the Laboratory of Plant Protection Department, Faculty of Agriculture and Science Building, Faculty of Mathematics and Natural Sciences, Hasanuddin University, Makassar.

The pathogen collected from infected cocoa pod was initially undertaken with reinoculation in the fresh pod wrapping plastic bag and placed in container until the symptom appeared. Once the symptom came out, a piece of symptomatic tissue was transferred onto V8 Medium with room temperature until colony grew. Isolates were then isolated and purified. After macroscopic and microscopic observation all isolates referred to *P. palmivora* were subcultured on slant agar and reserved in refrigerator for further tests. The isolate was grown onto V8 agar medium for 7 days. Pure cultures containing mycelium and sporangium were cut to a diameter of 0.3 cm, then inoculated in healthy cocoa fruit. The inoculation was undertaken by attaching a piece of agar containing 7-day-old sporangia and mycelium to the central part of the cocoa pod. Then, the agar pieces were covered with adhesive tape and incubated at room temperature for 7 hours. The parameter was presence of lesion on top of tissue. Identification and diversity analyze of *P. palmivora* molecularly was conducted firstly through DNA extraction using the method of Goodwin et al. [7] and Sambrook et al. [8]. Amplifying PCR product utilized universal primers of ITS4 and ITS5 [9]. The primary base composition of ITS4 is 5’-TCCTCCGCTTA TTGATATGC-3’ and primary ITS5 is 5’-GGAGTAAAAGTCA GCAAG-3’ [10]. Diversity between isolates was analyzed using BOXA1R primer (5’-CTACGGCAAGGCCGAC GCTGACG-3’). DNA fragments were amplified with gel electrophoresis (0.9%) and stained with ethidium bromide (0.5 µg/ml) for 30 minutes then rinsing with H2O. DNA fragment was observed above the UV trans illuminator and photographed with UV gel documentation.

3. Results and discussion

3.1. Isolation and identification of Phytophthora isolates

Twenty one (21) isolates obtained from across South Sulawesi consisted of Bone (12 isolates) from Bone (2 isolates from Itterung Village, 6 isolates from Ajjalireng Village and 4 isolates from Patangnga Village), 6 isolates from Bulukumba (5 isolates Karossil village and 1 Tanah Harapan village isolate and 3 isolates from Pinrang Padelo Village). The colonies of the isolates were dense compact-like with segregated flat edges, white color mycelium and forming a star-like pattern. Morphological hyphae shown that various types of hyphal swellings were exist within isolates. Hyphal swellings containing many nuclei (coenocytic) and no septate, generally were torulose, coralloid, smooth, coarse, regular, irregular and loops (unpublished data). They formed a branch point followed by a globular round swell with a thick cell wall called as chlamydospore. The morphology of sporangium and zoosporangium were observed by immersing 21 isolates of *P. palmivora* colonies with sterile water for 8-12 hours. Sporangia were produced in clusters sympodial, papillate and have ovoid form with the widest part close to the base. Detached sporangium contained a short pedicel and the time required to produce this sporangium of each isolate varies from 5-15 days. Oospores formed spherical and an amphigynous antheridia (figure 1). Oospores were found in all isolates from three regencies mentioned above but produced in a small number. Due to the growth of some isolates also were very slow and embedded into the medium, ten (10) best isolates with clear specific characters of *P. palmivora* representing of each regency were chosen, three isolates from Luwu and Pinrang respectively, and four isolates from Gowa regency for further determination using molecular marker. Based on morphological and microscopic features, all collected isolated referred to *P. palmivora*. 
Figure 1. Colony morphology of Phytophthora palmivora on V8 agar. Morphology of P. palmivora: stellate pattern (A), Ovoid sporangium of P. palmivora (B), Smooth globose oogonium of P. palmivora (C), oospores.

3.2. Virulence analysis of P. palmivora isolates

Virulence assessment of P. palmivora isolates was carried out through observations of the latent period, and disease development by measuring of symptom area on cocoa pods (table 1). The latent period of all twenty-one isolates ranged from 2-7 days. The fastest latent period was observed in BNL1P7 isolate, that is 2 days after inoculation. The results showed a virulence difference of P. palmivora isolates tested. According to Hiasinta [11], fusion between two different nuclei in mycelium (heterocariosis) can occur in P. palmivora which can cause genetic changes. The occurrence of epidemics of cocoa pod rot is also influenced by gene flow. P. palmivora has asexual propagules in the form of sporangium, zoospores, and chlamydospores. This asexual propagule is a chain of genes that have adapted and are selected for their fitness in a plant growth environment. Planting cocoa that is resistant to P. palmivora pathogens that are planted in large areas and the use of chemicals to control cocoa pod rot causes genetic stress in P. palmivora populations, thereby causing genetic changes of P. palmivora from avirulent to virulent.

Table 1. Infection rates and virulence degree of 21 Phytophthora palmivora isolates 3-7 day after inoculation on cocoa pod of S2 clone.

| Isolates   | Necrotic area on observation day (cm²) | Increasing average of necrotic area (cm²/ day) | Virulence degree   |
|------------|----------------------------------------|-----------------------------------------------|--------------------|
|            | 3                                      | 5                                      | 7                                      |                     |
| BN L1 P2   | 8.55                                   | 80.73                                 | 280.71                             | 56.14   | highly virulent |
| BN L1 P7   | 19.57                                  | 74.44                                 | 281.65                             | 56.33    | highly virulent |
| BN L2 P2   | 6.85                                   | 64.51                                 | 166.84                             | 33.37    | highly virulent |
| BN L2 P3   | 11.09                                  | 57.78                                 | 172.04                             | 34.41    | highly virulent |
| BN L2 P5   | 5.00                                   | 60.90                                 | 365.98                             | 73.20    | highly virulent |
| BN L2 P6   | 8.93                                   | 62.07                                 | 198.46                             | 39.69    | highly virulent |
| BN L2 P8   | 16.35                                  | 74.12                                 | 356.06                             | 71.21    | highly virulent |
| BN L2 P9   | 0.71                                   | 29.84                                 | 130.88                             | 26.18    | highly virulent |
| BN L3 P1   | 10.61                                  | 61.81                                 | 215.57                             | 45.11    | highly virulent |
| BN L3 P2   | 15.71                                  | 71.48                                 | 214.07                             | 42.81    | highly virulent |
| BN L3 P3   | 0.00                                   | 31.36                                 | 202.59                             | 40.52    | highly virulent |
| BN L3 P7   | 5.54                                   | 56.13                                 | 124.10                             | 24.82    | highly virulent |
| BK L1 P9   | 2.68                                   | 35.58                                 | 94.51                              | 18.90    | highly virulent |
| BK L2 P2   | 2.40                                   | 32.77                                 | 177.59                             | 35.52    | moderate virulent |
| BK L2 P3   | 3.40                                   | 33.73                                 | 147.04                             | 29.41    | moderate virulent |
| BK L2 P6   | 6.14                                   | 54.58                                 | 211.29                             | 44.26    | highly virulent |
| BK L2 P7   | 14.75                                  | 81.85                                 | 346.61                             | 69.32    | moderate virulent |
| BK L2 P9   | 0.38                                   | 25.19                                 | 125.17                             | 25.07    | highly virulent |
| PR L2 P6   | 0.48                                   | 85.12                                 | 230.03                             | 46.01    | highly virulent |
| PR L2 P7   | 6.58                                   | 65.00                                 | 247.95                             | 49.59    | highly virulent |
| PR L2 P8   | 10.43                                  | 56.32                                 | 170.31                             | 34.06    | highly virulent |
3.3. Molecular analysis of *P. palmivora* isolates

Amplification of twenty-one Phytophthora isolates resulted in a DNA band in size of 900 bp, whereas analyze using REP-PCR technique with BOXA1R primer, resulted 4-7 DNA bands in size of 250-1500 base pairs. Based on molecular analyze, six isolates have a monomorphic band pattern that produces bands at 500 bp, 600 bp and 700 bp sizes, namely isolates BNL3P1, BNL3P7, BNL2P9, BNL2P6, BKL2P3 and BNL1P7. Five isolates were from Bone and only one isolate (BKL2P3) was from Bulukumba. There were also one Bone isolates that have same banding pattern with Bulukumba isolate, namely BNL2P2 and BKL2P9. One isolate from Pinrang PRL2P8 had different banding pattern in comparison with other isolates (unpublished data). These results show that among the isolates tested had high diversity. Phylogenetic analysis showed that the genetic diversity of *P. palmivora* isolates was 67% (figure 2.). This result is different from the data obtained by Umayah et.al [12], that the genetic similarity of *P. palmivora* from cocoa is 83%. This difference can occur allegedly due to genetic differences from samples of *P. palmivora* isolates and the primer types used.

![Figure 2. Dendrogram of twenty-one isolates of *P. palmivora* species using Rep-PCR BOXA1R primer](image)

Classification of characters species within the genus Phytophthora evolved by several observation including colony morphology, characteristics of sporangium and oogonium, presence or absence of chlamydospores and hyphal swellings, as well as its physiological characters [13], isozyme patterns [14], and lately the combined use of molecular markers and morphological characteristics [15]. Electrophoretic protein banding patterns [16] and isozyme profiles [17] have been used to estimate intra- and inter-specific relatedness in Phytophthora of cacao. However, protein electrophoresis is often faced with insufficient resolution because of low variation in allozyme markers. Therefore, a molecular tool for identification is needed to complete the characterization of *P. palmivora* isolates. Differences in DNA profile fragment amplified by PCR can be used as a tool for distinguishing microbes at the level of genus, species and even specific genotype of the pathogen, or in the detection of pathogens, as well as to analyze variations between species in the rate of change of a single base [15]. Until now, a rapid identification and diagnosis of the major Phytophthora species (*P. palmivora*, *P. megakarya*, *P. capsici* and *P. citrophthora*) that cause pod rot of cacao worldwide remains a problem. A detailed analysis of the ITS regions of 161 isolates mainly received as *P. palmivora* and *P. megakarya*, including isolates from the less frequently reported species of *P. capsici* and *P. citrophthora*, predominantly from cacao in West and Central Africa was reported by Appiah [18]. Therefore, the morphological identification must be coupled with molecular character to obtain more accurate results.
4. Conclusion

There were 21 isolates obtained from three different areas in South Sulawesi shown to have similarity of *P. palmivora* species according to stellate pattern, ovoid sporangium, smooth globose oogonium and oospores. There were different levels of virulence among *P. palmivora* isolates tested, only four isolates had low virulence, the rest had high virulence level. Analysis of genetic diversity using a BOX A1R primer shown to have a high diversity (67%) among *P. palmivora* isolates.

References

[1] Direktorat Jendral Perkebunan 2016 *Perkembangan Produksi Komoditi Perkebunan 2015-2017* (Jakarta: Kementrian Pertanian RI).

[2] Gassa A, Fatahuddin, Abdullah T, Junaid M. 2016 Black ant (*Dolichoderus thoracicus*): Artificial diet and nest prospects in controlling cocoa pod borer (*Conopomorpha cramerella* Sn.). Research Journal of Pharmaceutical, Biological and Chemical Sciences. 7 3185-91

[3] Marelli J-P, Guest DI, Bailey BA, Evans HC, Brown JK, Junaid M, et al. 2019 Chocolate Under Threat from Old and New Cacao Diseases *Phytopathology* 109 1331-43

[4] Guest D 2007 Black pod: Diverse pathogens with a global impact on cocoa yield *Phytopathology* 97 1650-1653.

[5] Kuswinanti T, Junaid M, Melina, Surapati U, Ratnanawaty 2019 A promising microbial use on cocoa: decomposing cocoa waste and controlling Lasiodiplodia theobromae in-vitro. IOP Conference Series: Earth and Environmental Science 343:012256.

[6] Harni R, Taufiq E, and Amaria W 2014 Pengaruh formula fungisida nabati minyak cengkeh dan serai wanggi terhadap penyakit busuk buah kakao *J. Tanaman Industri dan Penyegar* 1 (1) 41-48.

[7] Goodwin S B, Drenth A, and Fry W E 1992 Cloning and genetic analysis of two highly polymorphic, moderately repetitive nuclear DNAs from Phytophthora infestans *Curr. Genetics* 22 107–115.

[8] Sambrook J, Fritsch E F and Maniatis T 1989 *Molecular Cloning: A Laboratory Manual*. Ed.2. (New York: Cold Spring Harbor Laboratory Press).

[9] Ristaino JB, Madritch M, Trout CL, Parra G. 1998 PCR Amplification of Ribosomal DNA for Species Identification in the Plant Pathogen Genus *Phytophthora* *Appl Environ Microbiol.* 64 (3):948.

[10] White T, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. San Diego: Academic Press; 1990

[11] Motulo H F, Sinaga M S, Hartana A, Suastika G, Aswidinnoor 2007 Karakter morfologi dan molekuler isolat *Phytophthora palmivora* asal kelapa dan kakao *Jurnal Littri* 13 111 – 118.

[12] Umaya A 2004 *Keragaman Genetik P. palmivora pada Kakao* *Jurnal Lembaga Penelitian dan Pengembangan* 1 (1) 41-48.

[13] Brasier C M, Griffin M J 1979 Taxonomy of Phytophthora palmivora on cocoa *Transaction of The British Mycological society* 72 111-143.

[14] Oudemans P, Coffey MD 1991 A revised systematic of twelve papillate Phytophthora species based on isozyme analysis *Mycol Res* 95 1025–1046.

[15] Kroon L P, Brouwer H, de Cock A W and Gover F 2012 The genus Phytophthora anno 2012. *Phytopathol. 102* (4) 348-364.

[16] Kaosiri T and Zentmyer G A 1980 Protein, esterase, and peroxidase patterns in the *Phytophthora palmivora* complex from cacao *Mycologia* 72 988-1000.

[17] Edel V 1998 *Polymerase chain reaction in mycology: an overview*. In Applications of PCR in Mycology eds. Bridge P D et al. (United Kingdom: CAB International) 1-20.

[18] Appiah A 2004 *Molecular analysis of the major Phytophthora species on cocoa* *Plant Pathology* 53 (2) 209 - 219.