An Early Detection of Latent Infection of *Ralstonia solanacearum* on Potato Tubers

Baharuddin (Corresponding author)
Department of Plant Pest and Disease, Faculty of Agriculture, Hasanuddin University
Makassar, 90245, South Sulawesi Indonesia
Tel: +62-411-587100 Fax: +62-411-587100 E-mail: *baharunhas@yahoo.com*

Tutik Kuswinanti,
Department of Plant Pest and Disease, Faculty of Agriculture, Hasanuddin University
Makassar, 90245, South Sulawesi Indonesia
Tel: +62-411-587100 Fax: +62-411-587100 E-mail: *koeswinanti@yahoo.com*

Ach Syaifuddin
Department of Plant Pest and Disease, Faculty of Agriculture, Hasanuddin University
Makassar, 90245, South Sulawesi Indonesia
Tel: +62-411-587100 Fax: +62-411-587100 E-mail: *aceha.syaif@gmail.com*

(Received: Sep 10, 2014; Reviewed: Sep 25, 2014; Accepted: Nov 24, 2014)

Abstract: *Ralstonia solanacearum* is not only a soil-borne pathogen but also a seed-borne bacterial pathogen. National Seed Agency require that seed-source (G0) must free from bacterial wilt infection (0%) in potato seed certification process. This study aimed to determine an early and accurately detection of *Ralstonia solanacearum* in potato seed-source production (G0) on aeroponics cultivation system, which previously treated with microbial antagonists and artificialy infected with *Ralstonia solanacearum*. Seed potatoes were obtained from the previous study. Antagonist isolates NS01, S04, G06, and NG02 were applied separately in aeroponic system in nutrient solution, then artificially infected with *R. solanacearum*. These four isolates is quite effective inhibite R. *solanacearum* infection in potato aeroponic system with the intensity attach of 5, 7, 9 and 21% respectively, compared to 100% in control. Although the symptoms of wilt disease were appeared in plants, but the seeds did not show typical symptoms. In the positive control that inoculated with *R. solanacearum* without antagonists, revealing immediately wilt and death of plants, 14 days after inoculation. The result of polymerace chain reaction (PCR) using OLI1 and Y2 primers on tubers without antagonist with 100% disease incidence showed, that all of the tuber have positive results indicated by the appearance of DNA bands in the size of 287 bp. From visually healthy seeds sample resulted from antagonist treatment, only few of them have positive result and mostly other tubers showed negative result. This means that the role of antagonists on suppression of wilt disease, cannot totally guarantee the tubers free from latent infection of *R. solanacearum*.

Keywords: *Ralstonia solanacearum*; latent infection; PCR; aeroponic; potato seed

1. Introduction

The main constrains to increasing potato production in Indonesia is the lack of good quality seeds, as well as the presence of plant pests and diseases. One of the important diseases in the potato crop is wilt disease caused by *Ralstonia solanacearum* (E.F. Smith) Yabuuchi, which cause losses of up to 80% and reduce the quality and quantity of potatoes (Aryanti *et al*., 2010).

Bacterial wilt can be effectively controlled by combining preventive measures, such as using healthy seed and crop rotation with non-host of bacterial wilt

i.e. carrots, cabbage and corn that able cut off the life cycle of pathogens. Rosida *et al.* (2009) utilized microbial antagonist's *Bacillus subtilis* and *Pseudomonas flourences* to prevent the incidence of bacterial wilt disease as "seed treatment".

An effort to increase potato production, especially through improved seed production technology. One healthy seed production technology that is being developed currently in Indonesia is the aeroponic system. Through this new system, G0 seed production can be multiplied up to 10 times compared to conventional systems, the seed size is 2-3 times larger, free of pests and pathogens, seed purity is preserved and can be produced any time, does not depend on the season (Baharuddin *et al*., 2012)

Problems that often arise in the seed production using aeroponic system are the lack of hygienist conditions in the greenhouse. Cuttings derived from tissue culture are pathogen-free seed, but it can be easily infected with pathogen and spread to other plants supported by a water circulation system and nutrients. The severity of wilt disease can reach up to 85%. Use of 2% chlorined water is a way to release water from pathogen contamination; however, the use of chlorined water will also turn off the microbial antagonists of these pathogens. Once pathogens contaminated in this system, it will immediately easy spread and infected to other plants (Lestari *et al*., 2010).

To avoid wilt disease contamination in aeroponic system, a research to develop antagonist agents that can protect potato plants and potato tubers from *R. solanacearum* infection has been conducted (Nurbaya *et al.*, 2011). Four anaerobic bacterial isolates, identified as *Clostridium* spp. have been tested their affectivity in potato aeroponic system that artificially infected with *R. solanacearum* (Nurbaya *et al*., 2013). To determine the presence of *R. solanacearum* in potato seed produced in these studies, more accurate detection technology using Polymerase Chain Reaction (PCR) is necessary to use.

R. solanacearum can spread through tubers (seed-borne) without causing symptoms (latent infection); therefore it is important to find out their presence in the seed. Moreover the National Seed Board, Department of Agriculture (2008) requires that the production of breeder seed G0 must be free from bacterial wilt disease (0%).

This study aimed to review the sensitivity and accuracy of PCR technique in detection of *R. solanacearum* in potato seed tubers especially in cases of latent infection (symptomless carriers) to support the healthy and high quality of potato seed production.

2. Materials and Methods

2.1 Source of Potato Seed

Seed potatoes were obtained from the previous study. Antagonist isolates NS01, S04, G06, and NG02 were applied separately in aeroponic system in nutrient solution, then artificial infected with *R. solanacearum* (Nurbaya *et al*., 2011). These four isolates is quite effective inhibite R. *solanacearum* infection in potato aeroponic system with the wilt disease incidences of 5, 7, 9 and 21% respectively, compared to 100% in control. Although the symptoms of wilt disease were appeared in plants, but the seeds did not show typical symptoms (Figure 1).

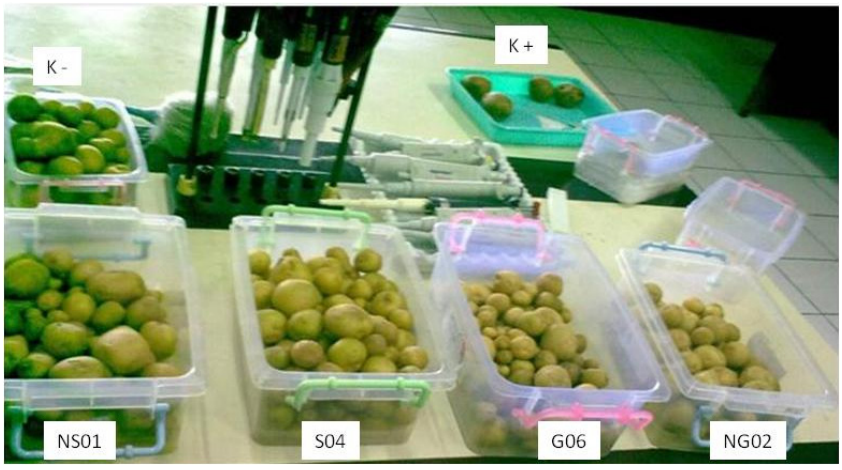

Figure 1. Seed potatoes samples produced from aeroponic system that previously treated with bacterial antagonists (NS01, S04, G06, and NG02 isolates) then artificially infected with *Ralstonia solanacearum*.

2.2 DNA extraction

Extraction of DNA samples from potato tubers following the working procedures of Silvere *et al.* (2007) and Kuswinanti *et al.* (2010) as follows: A total of 0.2 g of potato tuber samples were surface sterilized, cutted in small pieces then suspended with 0.5 mL of sterile distilled water in E-cup and then allowed to settle. After centrifugation, pellet was taken and added to 1 volume of Phenol: Chloroform: Isoamylalkohol (25: 24: 1). Suspension was vortexed for 1 min, then centrifuged at 10,000 rpm for 10 min, supernatant removed in a new tube, then added with 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of isopropanol. After thoroughly shaken and centrifugation at 10.000 rpm for 15 minutes. The supernatant was discarded, and the pellets were washed twice with 70% Ethanol. Ethanol then removed carefully and pellet let to dry on the block heater (40°C). Pellet then resuspended in 50 mL of TE (10 mM Tris - 1 mM EDTA pH 8.0) stored in refrigerator until use.

2.3 Amplification

A total of 25 µL PCR reaction consisting of 12.5 µL Top Taq Master Mix (Qiagen), 0.5 µL of each primer (10 pmol) consisting of OLI1 and Y2 primer (Seal, et al. 1993), 2 µL DNA template, 2.5 µL Corall load (Qiagen) and 7 µL nuclease free water. Amplification conditions consisted of: 1 cycle pre-denaturation 94^{o}C for 5 min, 35 cycles of denaturation 94^{o}C for 2 minutes, annealing 59^{o}C for 2 min, extension 72 °C for 30 seconds and a final extension at 72 ° C for 10 minutes.

2.4 Electrophoresis of Total DNA and PCR Products

Electrophoresis of PCR products using 1.8% of agarose gel, while for total DNA using a 0.8% agarose. PCR products were electrophoresed in a 2% agarose gel in 0.5xTAE solution (Tris Acetic Acid EDTA). A total of 8µl PCR product mixed with 2µl of loading dye, loaded in the gel electrophoresis wells, and runned at a voltage of 90 volts for 30 minutes. Documentation was conducted on UVP High Performance

Ultraviolet Transluminator (Biorad). The existence R. *solanacearum* on samples were characterized by the appearance of bands in size of 287 bp.

3. Results and Discussion

Polymerase Chain Reaction (PCR) using specific primers Y2 and OLI1 for *R. solanacearum* on seed potatoes, can detected in the presence of *R.solanacearum* both on visually symptomatic seed and on symptomless samples (Figure 2).

Figure 2. showed that on positive control, untreated seed samples that only infected with *R. solanacearum* caused 100% wilt disease incidence. The whole tuber samples showed positive results, indicated with appearance of DNA bands in the range of 287 bp. In other hand, negative control without R. *solanacearum* infection, no amplification was detected. In well 7-9, seed samples derived from NS01 antagonist treatment then infected artificially with R.*solanacearum* resulted wilt disease incidence of 5%. From three seed samples, two samples generated no bands and one sample generated bands in the size of 287 bp.

In contrast, the high incidence of wilt disease (21%) was obtained from NG02 antagonist treatment. Two of three seed samples (wells 10 and 12) gave positive band for *R.solanacearum*, while well 11 was negative. In treatment with S04 and G06, low disease incidence (7% and 9% respetively) was observed on plants and no symptom was detected on seeds. Two samples were uninfected and one sample generated positive amplification in each treatment.

Treatment of potato plants with bacterial antagonist following with bacterial wilt infection resulted lower disease incidence by visual observation. Using PCR method to detect the presence of *R. solanacearum* both in symptomless and symptomatic tuber indicated; that the use of single isolates antagonists has not been able give optimal protection against bacterial wilt. With a low disease incidence, it produces tubers apparently healthy, but can not guarantee the liberation of *R.solanacearum* in the tubers. The use of PCR with specific primers was able to detect the presence of bacterial

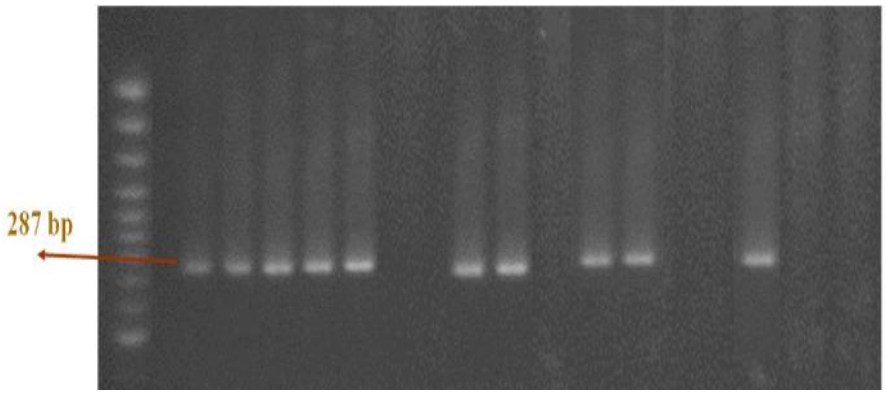

Figure 2. Amplification products of seed potatoes samples treated with antagonist and artificial inoculated with *R. solanacearum*: 1. Negative Control, 2: Positive control, 3-6: artificial inoculated samples with *R.solanacearum*, 7- 9: Treated samples with NS01 isolate then infected with *R.solanacearum*, 10-12: Treated samples with NG02 then infected with *R. solanacearum*, 13-15: Treated samples with S04 then infected with *R. Solanacearum,* 16-18: Treated samples with G06 then infected with *R. solanacearum*.

pathogens in very small concentration, to less than 5 picogram. It is much more accurate in comparison with the ELISA method. Seal *et al.* (1993), suggested that the PCR method is a rapid and sensitive method to describing a subgroup strains of *R. solanacearum* causes bacterial wilt.

The use of primers OLI 1 and Y2 were able to detect one cell of *R. solanacearum*. Furthermore Silvere *et al.* (2007) reported that with the same primer can proved the presence of *R.solanacearum* in potato tubers derived from naturally infected plants but showing no discoloration on the cutted tuber. In comparison with ELISA method, only 56% of tested tubers gave positive results whereas by PCR method 74% of tested tuber gave a positive result. Martins *et al*. (2000) tested the sensitivity of the system, and concluded that infected potato tubers with 3.7 x 10^3 CFU mL^{-1} concentration of *R. solanacearum*, recommends as a PCR standard procedure to detect the presence of *R.solanacearum* if its existence can no longer be confirmed by using selective media. PCR detection on potato seed derived from tissue culture propagation resulted no infection of *R.solanacearum* (Kuswinanti *et al.*, 2010). If the bacteria are actually carried on plantlets they may appear on the medium if the bacteria concentration is enough, it will also cause wilt symptoms on plantlets. The incidence of wilt disease in the aeroponic system, using cuttings derived from tissue culture, probably originate from contaminated water source comes from a locally opened irrigation or through contaminated equipment during transplanting process. Utilization of microbial antagonists singly in an aeroponic system to protect the potato seed from *R. solanacearum* infection. Further researchs are still needed to determine the optimal concentration of antagonists as well as the role of antagonist isolate either in singly or in combination to protect potato plants in the natural infection of *R.solanacearum*.

4. Conclusion

The use of Polymerase chain reaction using Oli1 and Y2 primers were able to detect the presence of *R. solanacearum* on symptomless seed potatoes (latent infection). Although the use of bacterial antagonist can reduce the incidence of wilt disease on potato plants but it can not totally guarantee that the potato seed tubers free from latent infection of *R. solanacearum.*

Acknowledgements

This study is one part of the National Strategic Research (PUSNAS) funded by the Ministry of National Higher Education-contract number 201/SP2H/PL/DITLITABMAS/2013 dated May 13th, 2013.

References

Aryanti E.L, T. Kuswinanti, Baharuddin. (2010). Isolasi dan Karakterisasi Mikroba Antagonis dari Rizosfer Tanaman Kentang Sistem Aeroponik yang Berpotensi sebagai Pengendali Penyakit Layu Bakteri *(Ralstonia solanacearum)*. J. Sain & Teknologi, 10 (1):74-86. (*in Indonesian*).

Baharuddin, Nurbaya, A.Syaifuddin. (2012). *Bertanam Kentang tanpa Tanah.* Buku 104 Inovasi Prospektif Indonesia. Business Innovation Center (BIC). Kementerian Riset dan Teknologi. (*in Indonesian*).

Direktorat Jenderal Hortikultura, Departemen Pertanian. (2008). Pedoman Perbenihan Kentang. 119 pp. (*in Indonesian*).

Kuswinanti T., A.Rosmana, A.Nasruddin. (2010). Optimalisasi Teknik PCR Untuk Deteksi Dini Bakteri Layu, Ralstonia *solanacearum*, pada Beberapa Varietas Benih Kentang. J. Fitomedika, 7 (2):115-118. (*in Indonesian*).

Martins O.M., K.Rudolph. (2002). Variability of *Ralstonia solanacearum* Strains Using Repetitive Extragenic Palin-Dromic Sequences. Bacterial Wilt Newsletter 17: 4-6.

Nurbaya, T. Kuswinanti, Baharuddin. (2013). Bacterial Antagonist Isolates In Controlling Bacterial Wilt Disease of Potato in Aeroponic Cultivation System. International Journal of Agriculture Systems, 1(1):49-54.

Nurbaya, Zulfikar, T. Kuswinati and Baharuddin. (2011). Kemampuan Mikroba Antagonis dalam Mengendalikan *Ralstonia solanacearum* pada Sistem Budidaya Aeroponik Tanaman Kentang. J. Fitomedika. 7 (3):155-158. (*in Indonesian*).

Rosida N., Baharuddin, A. Saranga. (2009). Keefektifan Mikroba Antagonis dalam Melindungi Bibit Kentang terhadap Penyakit Layu Bakteri (Ralstonia *solanacearum*). Prosiding Seminar Nasional Pekan Kentang (2008). Departemen Pertanian. 227-235. (*in Indonesian*).

Seal S.E., L.A. Jackson, J.P.W.Young and M.J. Daniels. (1993). Differentiation of *Pseudomonas solanacearum, Pseudomonas syzygii, Pseudomonas pickettii* and the Blood Disease Bacterium by partial 165 rRNA Sequencing: Construction of Oligonucleotide Primers For Sensitive Detection by Polymerase Chain Reaction. J. Gen. Microbiol. 139: 1587-1594.

Silvere J.R.P., V. Duarte, M.G. Moraes., C.A. Lopes., J.M.V. Fernandes, , J.L.N. Barni & Maciel. (2007). Epidemiological Analysis of Clones and Cultivars of Potato in Soil Naturally Infested with *Ralstonia solanacearum* biovar 2. Fitopatologia Brasileira 32:181-188.

Swanson J.K., L. Montes, L.Mejia and C. Allen. (2007). Detection of Latent Infections of *Ralstonia solanacearum* race 3 biovar 2 in geranium. Plant Dis. 91:828-834.

***