Biological control of solanaceous plant bacterial wilt by seed coating

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INTRODUCTION

Bacterial wilt caused by bacteria *Ralstonia solanacearum* is one of the important disease of Solanaceous crops (Kelman, 1953). The disease is difficult to control due to variability of the pathogen and complexity of entry point of the pathogen to the host plant. Moreover, the pathogen has a lot of host plants and could survive in the soil for prolong period, make it difficult to control by cultural methods (Hayward, 1991). Biological control based on the antagonism between rhizosphere bacteria and bacterial plant pathogen could be an effective method to reduce pathogen population and to reduce diseased plants (Cook and Baker, 1983). A strain of *Pseudomonas putida* (Pf20) isolated from the rhizosphere of *Mimosa invisa* proved could suppressed the growth of *R. solanacearum in vitro*, reduce wilt disease index in greenhouse and field experiments (Arwiyanto and Hartana, 2001). These results were obtained when the root system of seedlings were soaked in the water suspension of Pf20 before planted in the infested soil.

The method was laborious and need a lot of bacterial suspension. Therefore, we delivered the bacterial antagonist into the plant roots by coating the seed with inorganic materials and the antagonist itself. The results indicated that the antagonistic bacteria could survived in the coated seed but the population could not be maintained in high level for more than 4 weeks. Seedlings from coated seed containing the antagonistic bacteria have disease index significantly lower than control when challenged with a virulent population of *R. solanacearum*.

MATERIALS AND METHODS

Bacterial culture. A strain of *Pseudomonas putida* (Pf20) was taken from stock culture of the Laboratory of Plant Bacteriology, Faculty of Agriculture, Gadjah Mada University. The strain then was grown in the surface of King's B medium for 48 hours at room temperature. A fluorescent single colony of bacteria was picked up and grown in the slant agar of King's B medium. The culture was incubated further for 24 hours at room temperature. Spontaneous mutation of *P. putida* ($Pf20^{Nal\ Rif}$) was generated in King's B medium supplemented with 100 ppm of Nalidicix acid and 100 ppm Rifampicin.

An isolate of *R. solanacearum* (Rs47) was used as a challenge strain. The bacteria was taken from stock culture and grown in the surface of YPA (yeast extract 5g, peptone 10g, agar 15g, distilled water 1000ml, pH 7.2) medium for 48 hours at room temperature. A single white fluidal colony was picked up and grown in the slant agar of YPA medium and further incubated at room temperature for 24 hours.

Plant seed. Tobacco seeds were used as a model for seed coating. The seeds were disinfested with 0,5% NaOCl then air dried before used. Germination test was performed before coating.

Coating materials. Several coating materials were used in any combination. Clay, kaolin, and talc, SP36 (commercial-phosphorus fertilizer) were separately grinded and sieved until the size was 90 µm then sterilized by heating at 100°C for 4 hours. $CaCO3$ was grinded but not sterilized. One gram of sucrose was dissolved in 100 ml distilled water then sterilized by autoclaving at 121C for 10 seconds.

Coating method. The seeds were put in a petridish and shaken gently to disperse them. A bacterial suspension of *P. putida* in 1 % sucrose then was sprayed onto the seed surface followed by adding with coating materials. The mixture was pressed gently with a brush then the brush was whirled until the matrix coat the seeds. The process was repeated until the diameter of coated seed in the range of 1.5-2 mm. The coated seeds were taken and air dried.

Determination of fluorescent pseudomonads in coated seed. Survival of the fluorescent pseudomonads in the coated seeds were assayed immediately after coating and weekly afterward for 4 weeks as follow. A coated seed was plunged into a test tube containing 1 ml of a pH 7.0 phosphate buffer and shaken vigorously. After still for 5 minutes, the supernatant was tenfold serially diluted with the same buffer. At certain level of dilution, 100 µl of the suspension was spread onto the surface of King's B medium supplemented with 100 ppm of Nalidicix acid and 100 ppm Rifampicin. After incubation for 48 hours at room temperature, the fluorescent colonies were counted.

Disease suppression experiment. Coated seeds were sowing in the sterile soil. After 40 days, the seedlings were transplanted into the vinyl pot containing soil infested with *R. solanacearum*. The disease intensity was recorded at 40 days after transplanting.

RESULTS AND DISCUSSION

$Pf20^{Nal\ Rif}$ was not significantly different with the wild type (Table 1). The colonies of both strains were smooth, fluidal, and fluorescence in King's B medium and could grow in minimal medium. The generation time and its ability in suppressing *R. solanacearum* (Rs47) was not significantly different. It means that $Pf20^{Nal\ Rif}$ could be used as a representative of the wild type through out the experiments.

No	Traits	Bacterial strain	
		Pf20	$Pf20^{Nal\ Rif}$
1	Generation time*	35.8	38.8
2	Inhibition zone**	26.4	24.5
3	Growth in minimal medium	Yes	Yes
4	Fluorescence in King's B medium	Yes	Yes

Table 1. Traits of $Pf20^{Nal\ Rif}$ compared with the wild type
*** : in minutes**
**** : against RS47 (in mm)**

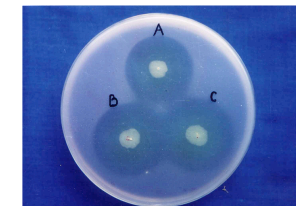

Figure 1: Suppression of Rs47 by Pf20 in King's B medium

In the formulation without Calcium carbonate (no 1, 2, and 3), population of $Pf20^{Nal\ Rif}$ only detected after coating while in the formulation with Calcium carbonate (no 4, 5, and 6), the bacterium could survive until 4 weeks but when the initial population is high (Figure 2). When the initial population was 10^7 cfu/ml and 10^6 cfu/ml, the bacteria could survived in the coated seed for two weeks after coating. The presence of calcium carbonate in the formulation of coating materials

might changed the pH into suitable range of bacterial growth. The pH range of *P. putida* Pf-20 is 5-10 (Widyastuti, 2001). Due to the limited availability of nutrient in the coated seed, the bacteria could not survive longer than 4 weeks after coating. In addition, pseudomonads fluorescent coated on seeds, have a short shelf life at room temperature, and are readily killed by desiccation (Hebbar and Lumsden, 1999). It is necessary to improve the coating materials by addition of substance that could protect the bacteria from desiccation or the coated seeds should be sown immediately after coating.

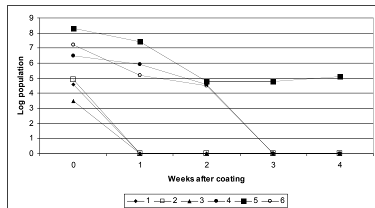

Figure 2. Population dynamic of *Pf20*$^{Nal\ Rif}$ in several coated seed

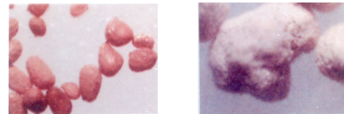

Figure 3: Original seed (left) and coated seed (right)

Bacterial wilt disease suppression

Bacterial wilt disease could be suppressed by Pf20 coated on the seed. At 40 days after transplanting, the disease index in the plot planted with seedlings from coated seed containing Pf20 were significantly lower compare with control (Table 2). Seedlings from coated seed without Pf20 (treatment no 7) could be attacked by the pathogen and the disease index was not significantly different with seedlings from uncoated seed (no 8). It means that Pf20 could be delivered into the rhizosphere by seed coating and protect plants from infection of *R. solanacearum*. Further experiments should be performed to determine the population of Pf20 and the pathogen in the rhizosphere. This kind of experiment could predict the mechanism involved. It is possible that Pf20 dominate in the rhizosphere and the pathogen was suppressed so that the population is lower for inciting disease, then it is presumed that the mechanism is antibiosis. Other mechanism is presumed to be induced resistance when both bacteria are in high population.

Treatment*	Disease Index**
4	16.6ab
5	11.6a
6	23.3b
7	63.3c
8	78.3c

Table 2. Disease index in the plot planted with seedlings from coated seeds
*** the number is for coated seed as in figure 2, except no 7 (coated seed without Pf20) and no 8 (uncoated seed)**
**** Value represent the average of three replications**
***** Means in a column followed by the same letter are not significantly different**

CONCLUSIONS

P. putida Pf20 could protect plant from bacterial wilt by seed coating application. The mechanism of disease suppression is yet not known and need further investigation.

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