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Development of the New Methods for Ecological Study of Soybean Bacterial Pustule : An Artificial Inoculation Method of Soybean Seed with *Xanthomonas campestris* pv. *glycines* for Inducing Disease Expression

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ABSTRACT

An attempt was made to develop the artificial inoculation method of seed transmission experiments. Injection of *Xanthomonas campestris* pv. *glycines* through seed mycopyle at 10^3 and 10^5 cfu/seed gave 100% seed transmission with no effect on seed germination. When seeds derived from artificially inoculated soybean plants were used, the rate of seed transmission was dependent on the disease severity of parent plants. Seed transmission ratio was higher with seeds taken from plants inoculated 3 times than those inoculated 2 and 1 times respectively. The administration of the vacuum pump (20 lb/inch^2) for 10, 20 and 30 min after soaking seeds in bacterial suspenssion at 10^6 cfu/ml revealed no difference in percent seed transmission. Furthermore, this method significantly reduced seed germination.

Key words : *Xanthomonas campestris* pv. *glycines*, soybean (*Glycine max*), bacterial pustule, seed transmission

INTRODUCTION

Bacterial pustule caused by *Xanthomonas campestris* pv. *glycines* (Nakano) Dye, is one of the most important diseases of soybean (*Glycine max*) planted in central region of Thailand. The losses due to this disease is considerably high whenever recommended variety, SJ, commonly grown around the country is heavily infected (Prathuangwong and Amnuaykit, 1987). Transmission through soybean seed of this pathogen has been reported with 8% where the causal pathogen was present in cotyledon as well as in seed coat of mature seed, but the bacterium was absent from the embryo (Prathuangwong and Choochoa, 1988). However, in some studies, bacterial pustule severely occurred in field conditions and seed inspection after harvesting indicated that percent seed transmission was less than it should be, probably because of unsuitable detection methods. Another possibility was that the amount of causal pathogen was too small to be detected. Therefore, in the experiment concerning seed transmission, if the seeds were heavily infected, the seed investigation has higher chance to be successful. The objective of this study is to determine the method inducing disease expression of bacterial pustule from soybean seeds so that a reliable seed test could be established.

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MATERIALS AND METHODS

The transmission or infestation of *X. campestris* pv. *glycines* through soybean seeds was tested using strain *Xcg*-039 with a marker of 50 ppm polymyxin B sulfate resistance. The details of the methods were as the followings:

Foliage inoculation

The inoculation tests were conducted by planting SJ4 soybean variety at the experimental plots of KURDI KampaengSaen, Nakhon Pathom with plant spacing of 50 × 25 cm. CRD was employed in the experimental design with the following 4 treatments: 1) Inoculating bacterial suspension 1 time when soybean was 20 days old, 2) Inoculating bacterial suspension 2 times when soybean were 20 and 30 days old, 3) Inoculating bacterial suspension 3 times when soybean were 20, 30 and 40 days old, and 4) No inoculation of bacterial suspension (control). There were 3 replications in the experiments. At each inoculation, the plants were placed under favorable conditions of infection by mist spraying 1 day before inoculation. The bacterial suspension was prepared by culturing *Xcg*-039 on nutrient glucose broth for 28 h and diluting the 100 ml bacterial culture into 400 ml water obtaining the approximate concentration of 10^8 cfu/ml. Fifty grams of 600 mesh carborundum and 1 teaspoon Triton CS-7 were added per liter and shaken. The mixture was then poured into atomized pressure knapsack sprayer with 10-12 lbs/inch^2 pressure for spraying (Prathuangwong, 1984). After soybean became 50 days old, the severity of bacterial pustules was evaluated by sampling the 6th trifoliate leaf of plants from the two middle rows at each replicate in every other hole except the first and last holes for lesion count per leaf following method given by Vichitrananda (1975). After harvesting, 100 seeds from each replicate were sampled, and % seed transmission was determined by agar plating method with semiselective medium, MXG(Modified Medium for *Xanthomonas campestris* pv. *glycines*) for *X. campestris* pv. *glycines* (Prathuangwong *et al*., 1997) plus with 50 ppm polymyxin B sulfate. Plates were kept at room temperature and were checked throughout the week by dropping iodine solution on the MXG. If clear zones were noticed to encircle the seed, the seed was judged to be infected with the bacterial pustule pathogen. The result was recorded by counting the number of infected seeds and calculating % seed transmission.

Seed inoculation by injection

The artificial injection of bacterial suspension into the seed was made at 3 concentration levels of 1.3×10^5, 1.3×10^3 and 1.3×10 cfu/seed with the control of sterile distilled water and no injection of seeds.

SJ4 soybean seeds were washed in sterilized distilled water added a few drops of Tween 20, and stirred for 20 min. Seeds were washed with sterilized distilled water 3 times and dried (seeds still not hardened). With sterile 10 ml injection pipette (Hamilton), 1 ml of *Xcg*-039 suspension at each concentration was injected into micropyle of each seed and these seeds were dried. After that, injected seeds were germinated by the between-paper method with 4 replications using 100 seeds per one replication. These seeds were incubated at 25-30° C for 7 days and then the percentage of germination was checked (ISTA, 1976).

A part of injected seeds was used for assaying the ratio of infected seeds and seedling symptom with *X. campestris* pv. *glycines*. Each 100 injected seeds with bacterial suspensions at different concentrations was placed on MXG, the semiselective medium coupled with 50 ppm polymyxin B sulfate and planted in the greenhouse. The results were recorded as in foliage inoculation.

Seed inoculation by soaking under vacuum

Seeds were soaked in bacterial suspension and subjected to vacuum for artificial infiltration. The test seeds were surface disinfected and then dried. Two hundred seeds were soaked in 200 ml bacterial suspension at the concentration of 2.21×10^6 cfu/ml (ratio of 1:1). One to two drops of Triton CS-7 was added to the suspension. After that, seeds were put in the vacuum pump with pressure of 20 lbs/inch^2 for 10, 20, 30 min and dried. The rate of germinated and infected seeds in percent were also checked as in foliage inoculation.

RESULTS

Foliage inoculation

The bacterial pustule pathogen was inoculated on the plants and disease severity was assayed when soybean was 50 days old at R3 growth stage. The results revealed that the disease severity was at 3 statistical differences. The plots sprayed with bacterial suspension 3 and 2 times showed no difference from one another with the average number of lesions of 4.45 and 3.80 per leaf respectively (Table 1). This was, however, significantly different from the plots sprayed with the suspension only 1 time as well as the non-sprayed control with the average number of lesions of 2.69 and 0.98 per leaf respectively. There was also significant difference in % seed transmission in every treatment: the plots sprayed with bacterial suspension 3 and 2 times and the control, % seed transmission being 48.67, 33.17 and 5.17 % respectively. Percent seed transmission (Y) received from the inoculated plants depended on the rate of bacterial pustule severity (X) with a linear regression equation: $Y = 1.1462 + 0.0731X$ with correlation coefficient (r) = 0.9452.

Seed inoculation by injection

The injection of the bacterial suspension into seeds at the concentration of 1.3×10^5, 1.3×10^3 and 1.3×10 cfu/seed revealed that there was no significant difference in % seed infection which were 100, 100 and 97% respectively (Table 1). The difference was found from both controls of seeds injected with and without sterilized distilled water. When % seed germination was compared, however, no significant difference was found in every treatment, or no loss was observed in % seed germination from both controls. Recovery of *X. campestris* pv. *glycines* from artificially injected seeds with 1.3×10^5 cfu/seed revealed the large clear zone of pathogen appearance encircled those contaminated ones. Seeds injected with and without sterilized distilling water were however, void of such area on MXG after dropping with iodine solution (Figure 1). When injected seed was planted in the greenhouse, symptom of small white pustule raised up from the cotyledon surface of seedling appeared in 6 days after sowing (Figure 2).

Seed inoculation by soaking under vacuum

The inoculation of soybean seeds by soaking in the bacterial suspension and then putting under vacuum at pressure of 20 lbs/inch^2 for 10, 20 and 30 min revealed the statistical difference in the % seed infection. Seeds vacuumed in bacterial suspension for 20 and 30 min showed the highest % seed infection of 98 and 97 % respectively, and for 10 min only 85% (Table 1). The control, vacuumed and unvacuumed in sterilized distilled water showed no seed transmission at all. As for the seed germination, seeds vacuumed either in bacterial suspension or in sterilized distilled water showed the decline in seed germination, and the difference was statistically significant (Table 1).

DISCUSSION

Different experimental methods were employed for assaying the seed transmission of *X.*

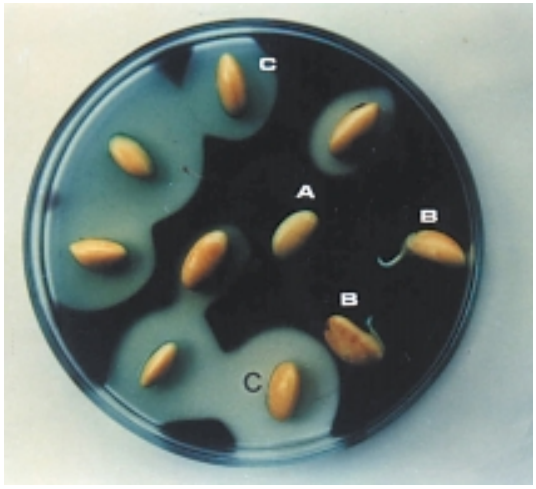

Figure 1 Recovery of *Xanthomonas campestris* pv. *glycines* from artificially injected seeds with 1.3×10^5 cfu/seed showed clear zone around injected seeds (C) after adding of iodine solution on modified medium for *Xanthomonas campestris* pv. *glycines* (MXG). A and B are the seeds injected with and without sterilized distilled water.

Figure 2 Seedling symptom of soybean bacterial pustule from planting seed injected with *Xanthomonas campestris* pv. *glycines* at 1.3×10^5 cfu/seed. c = cotyledon, h = hypocotyle, s = symptom of small white pustule lesion raised up from the cotyledon surface.

campestris pv. *glycines*. Plant inoculation of the pathogen was made for inducing bacterial infection to seeds. The results revealed that the bacterial pustule severity of soybean foliage correlated with % seed infection of soybean seeds. As the severity of foliage bacterial pustule increased, the ratio of infected seeds also increased. In this experiment, the control seeds also showed low incidence of infection. This was probably because the seeds used for experiments had already been naturally infected with the pathogen. Numbers of inoculation caused the different levels of severity to the plants as well since the growth stages of host plant at inoculation were partly responsible for such difference (Prathuangwong, 1983). The rate of bacterial pustule severity of soybean were correlated with % seed transmission of the pathogen to soybean seeds. When these seeds were planted, howevr, the percent infestation of seeds revealed no correlation with the rate of foliage infection. It was probably due to the fact that the infected seeds after planting could develop disease symptoms at different severities depending on several factors such as the temperature and relative humidity. In addition, the production of pathogen or infected seeds, the number of infected seeds in seed samples, wind, rain, and insects which facilitate the severity of *X. campestris* pv. *glycines* were also responsible for the disease development.

The injection with 2 levels of bacterial suspension at 10^3 and 10^5 cfu/seed into the seeds revealed 100% seed infestation while seed germination ratio was not destroyed. However, the three bacterial concentrations of 10^5, 10^3 and 10 cfu/seed were not significant in seed infestation but at 10 cfu/seed, the seed transmission was not observed. Uematsu *et al*. (1983) also reported that the % seed germination did not decrease at 20 to 10^5 cfu/seed and at 10^5 cfu/seed, % seed transmission of the pathogen to the seedling was relatively high with disease occurrence of 53.65 %

Table 1 Seed germination and transmission of *Xanthomonas campestris* pv. *glycines* infected soybean seeds by different methods of inoculation[1/].

Method of inoculation	Bacterial density (cfu/ml)	Accessions	Disease severity (lesions/leaf)	Seed transmission (%)	Seed germination (%)
Foliage spray	1×10^8	3[2/]	4.45 a	48.67 a	-
		2	3.80 a	33.17 a	-
		1	2.69 b	13.33 c	-
	Control[3/]		0.98 c	5.17 d	-
CV(%)			12.88	19.4	-
Seed injection	1.3×10^5 [4/]	-	-	100 a	86
	1.3×10^3	-	-	100 a	88
	1.3×10	-	-	97 a	85
	Control[5/]	-	-	0 b	87
	Control[6/]	-	-	0 b	84
CV(%)		-	-	5.32	2.00
Seed infiltration	2.21×10^6	10[7/]	-	85 a	59 a
		20	-	98 a	50 a
		30	-	97 a	55 a
	Control[8/]	10	-	0 c	53 a
		20	-	0 c	50 a
		30	-	0 c	54 a
	Control[9/]	0	-	0 c	80 b
CV(%)			-[10/]	17.32	4.07

[1/] Means in each vertical section of each inoculation method followed by the same letter do not differ significantly at P = 0.05, according to Duncan's multiple range test.
[2/] Time of inoculation at 3, 2 and 1.
[3/] Plants were sprayed with distilled water.
[4/] Colony forming unit per seed with 3-level concentrations of the pathogen.
[5/] Seeds were injected with distilled water.
[6/] Seeds without injection.
[7/] Time of vacuum infiltration with 3 levels of 10, 20 and 30 min.
[8/] Seeds were soaked in distilled water before vacuum infiltration.
[9/] Seeds without vacuum infiltration.
[10/] Not determined.

(Uematsu *et al*., 1983).

The soaking of seeds in the bacterial suspension at 2.21×10^6 cfu/ml followed by vacuum at pressure of 20 lbs/inch^2 for 10, 20 and 30 min compared with the control of seeds soaked in sterilized distilled water with or without vacuum treatment revealed that there was no difference in % seed infestation vacuumed for 20 and 30 min of 98 % and 98% respectively. In seeds vacuumed for 10 min, the % seed infestation was reduced to 85 %. Such vacuum method was also found to reduce % seed germination in comparison with the unvacuumed control.

The investigation on inducing diseased seeds in relation to bacterial pustule severity through seed transmission and the injection of the bacterial suspension at 10^5 cfu/seed should be applied since the pathogen could be transmitted through seeds at 100 %. In addition, at such bacterial density, % seed transmission of the causal pathogen to the seedling was relatively high without reducing % seed germination. This result is however, necessary for reliable determination of experiments concerning infected seeds.

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