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A Detached Leaf Culture Technique for Resistance Screening Against Bacterial Pustule in Soybean

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ABSTRACT

A series of experiments was conducted to obtain information on variation in virulence of *Xanthomonas campestris* pv. *glycines*, the causal organism of bacterial pustule, resistance of the soybean (*Glycine max*) host, and methods of screening for resistance. Variation was found in both virulence and resistance. It was concluded that, given the levels of variability detected, use of a detached leaf technique with inoculation by a pinprick method would be the most effective means of selecting for resistance.

Key words : disease resistance, screening technique, *Xanthomonas campestris* pv. *glycines*

INTRODUCTION

Bacterial pustule, caused by *Xanthomonas campestris* pv. *glycines* (Nakano) Dye, is a serious disease of soybean (*Glycine max* (*L.*) *Merrill*) in Thailand, particularly in wet season crops (Chaisit, 1988). Symptoms of bacterial pustule disease include dark brown necrotic spots, and extensive chlorosis of the leaves. As the disease progresses, abscission of affected leaves reduces the photosynthetic area of the plants with a consequent reduction in seed yield (Sinclair and Backman, 1989).

The popular Thai cultivars SJ 2 and SJ 5 are susceptible while recently developed cultivars such as Sukhothai 1 are resistant (Surin, 1988). Many American cultivars are resistant with resistance being attributed to a single recessive gene (Hartwig and Lehman, 1951).

Although it has been reported that there is no pathotype or race development in the bacterial pustule pathogen (Patel *et al.*, 1972; Jindal *et al.*, 1981; Hokawat and Rudolph, 1988), Jainkittivong *et al.* (1989) concluded from a study of six pustule isolates on 45 soybean genotypes that there are pustule strain differences. Jainkittivong *et al.* (1989) classified the soybean genotypes into nine groups. American cultivars Clark 63 and Harosoy and an accession from Africa (TGX297-192C) were resistant to all strains isolated from Thailand but were susceptible to strain S12 from Japan. SJ4, SJ5 and cv. Akazaya were susceptible to all strains. They concluded that resistance is possibly controlled by a number of genes.

Selection for resistance of soybean to bacterial pustule in Thailand has mostly been based on natural infection in the field where effectiveness of selection depended on the occurrence of seasonal conditions that promoted disease development. Disease severity can vary between geographic locations and seasons. Expression of resistance might also be expected to vary with the prevalent strains of the pathogen at different locations. Screening in the field for resistance may also be complicated by the interaction between bacterial pustule and other diseases such as anthracnose such that observed symptoms cannot be reliably ascribed to either pathogen. Consequently, field screening for resistance must be very extensive if a reliable assessment of resistance is to be obtained.

In order to improve the efficiency and effectiveness of selection for resistance, studies were undertaken to develop a technique based on inoculation of detached leaves with identified strains of the pathogen under controlled conditions in the laboratory.

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MATERIALS AND METHODS

Collection and storage of pathogen isolates

Leaves were collected from infected soybean plants in plots of twelve cultivars grown at eight locations (Table 1) and transported to the laboratory over ice in an insulated container. Diseased samples were isolated for the pathogen on semisynthetic potato agar by standard procedures. A total of 40 strains of *Xanthomonas campestris* pv. *glycines*, including four from Nakhon Sawan, seven from Chiang Mai, eight from Phra Phuttabhat, seven from Khon Kaen, eight from Sukhothai and six from Kamphaengsaen were collected and stored under parafin oil in a refrigerator.

Culture of detached soybean leaves

Leaves were excised at the base of the petiole from plants grown in a glasshouse and free from disease symptoms. After excision, the petioles were immediately wrapped in moist cotton wool, the leaves were inoculated and placed on moist paper in shallow plastic trays. The trays were covered with transparent lids and placed on shelves under fluorescent lights. There were two daylight 40w fluorescent tubes above each shelf. The shelf unit was located in an airconditioned laboratory where the temperature was maintained around 28°C. Leaves did not wilt or discolour under these conditions for at least two weeks.

Inoculation procedures

Inoculum was prepared by suspending the bacterial culture in sterilised distilled water to give a bacterial concentration of about 1 X 10^{10} cfu/ml.

i. Whole plants. Carborundum (600 mesh) was added to the inoculum at the rate of 1 g/l and the mixture sprayed onto both the upper and lower surfaces of leaves with an adjustable pressure vacuum pump at 15 psi until the leaves were completely wet. Plants were covered for 48 hours with moistened plastic sheet.

ii. Detached leaves. Two methods of inoculation were used:

a. A 'cotton bud' or small wad of cloth wrapped around a dissecting needle was dipped in the inoculum and carborundum mixture and wiped across the leaf surface. This was repeated until the whole of the upper leaf surface was moistened.

b. A grid of 16 pins embedded in a cork base was dipped in the inoculum and used to prick the leaf surface. Four sets of pin pricks were made in each leaf. After inoculation, detached leaves were cultured as described above.

Disease susceptibility scores

In the field, disease severity was recorded on a five point scale where:

0 = no symptoms
1 = 1-5% leaf surface affected
2 = 6-10% leaf surface affected
3 = 11-25% leaf surface affected
4 = > 25% leaf surface affected and leaf fall

On whole plants and detached leaves that were inoculated with the inoculum solution containing carborundum, infection was recorded as the number of pustules developed after two weeks.

On detached leaves which were inoculated by pinprick, symptoms developed rapidly and the number of pustules could be counted after three days (Figure 1).

Experiments

Field survey of pathogen occurrence and host genotype reaction

Twelve soybean genotypes were grown at eight locations (Table 1) during the rainy season in 1986. At each location, each genotype was sown in a single plot of size 1 x 3 m. Spacing between rows was 50 cm and within rows, hills of two plants were spaced 10 cm apart. A mixed fertiliser was applied before planting to provide 3 kg N, 9 kg P and 6 kg K per rai. Plots were hand weeded and sprayed with insecticide as required.

Plant growth stage (Fehr *et al.*, 1971) and pustule severity were recorded at 10-15 day intervals during crop growth.

Effect of plant growth stage on expression of disease symptoms

Two experiments were conducted:

a. Cultivars SJ 5 and Sukhothai 1 were grown in pots in a glasshouse and the uppermost leaf inoculated at 21, 28, 35, 42, 49 days after sowing. An equivalent leaf was detached from other plants and inoculated by the wipe method as described above.

b. Leaves of cultivar SJ 5 at different growth stages as described by Fehr *et al.* (1971) were inoculated either by spraying whole plants or by treating individually after being detached. Detached leaves were inoculated by either the wipe method or by pricking with a grid of needles.

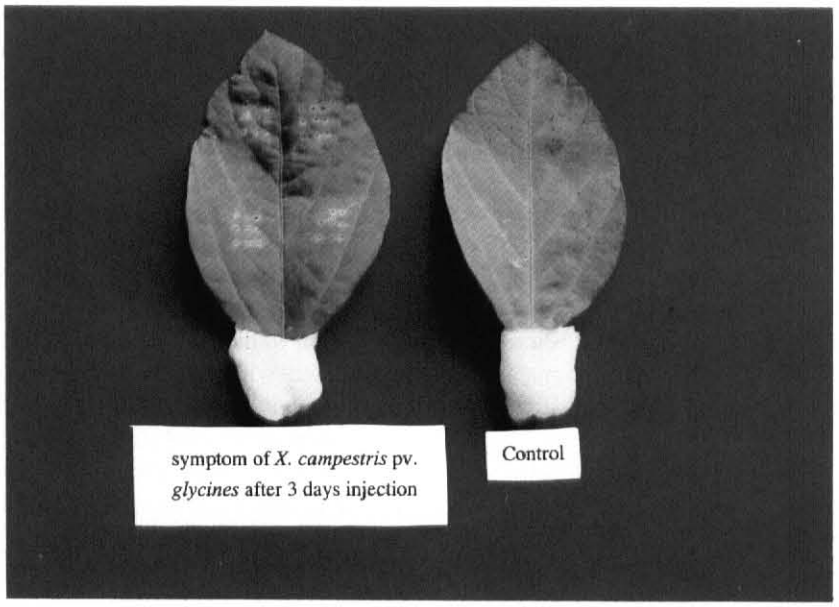

Figure 1 **Pustule symptom development on leaves of SJ 4 three days after inoculation with *Xanthomonas campestris* pv. *glycines* by the pinprick method.**

Interaction between soybean genotypes and pathogen isolates

Five strains of the pathogen, collected from different locations in Thailand (Table 4), were separately inoculated onto six genotypes (Table 4) using the pinprick method on detached leaves in order to illustrate variability in pathogen virulence and host resistance.

RESULTS

Variation in disease reaction in the field

The results presented in Table1 for disease reaction of twelve varieties grown at eight locations show the extent of variation that can occur. The more susceptible vaieties such as SJ 1, SJ 2, SJ 4 and SJ 5 recorded scores from 0 to 4 at the different locations and had mean scores between 2.0 and 2.5. The more resistant varieties such as Sukhothai 1, AGS 269 and G 8190 had scores from 0 to 2 with means of < 1.0. Similarly, there was variation between locations for which mean scores ranged from 0.3 to 2.7.

Variation in disease reaction with growth stage

More pustules formed on leaves of plants inoculated at 35 and 42 days after sowing than on leaves of plants inoculated at other times (Table 2). This time of inoculation was equivalent to inoculation of leaves at the V3 and V4 growth stages on which more pustules were formed than on leaves at other stages (Table 3). This same relationship was observed regardless of inoculation method. The number of pustules that developed with the pinprick method was much higher than following leaf wiping and thus the pinprick method had a potential to provide better discrimination between resistant and susceptible varieties (Table 3).

Interaction between pathogen isolates and soybean genotypes

The data in Table 4 shows that there was considerable variation in virulence among strains of the pathogen and in resistance among genotypes of the host. Strain 014 was highly virulent and all varieties could be considered susceptible to this strain. Strain 101-1 was highly avirulent and all varieties were quite resistant to this strain. The other strains elicited some variation in reaction of the varieties. For example, Sukhothai 1 and Nakhon Sawan 1 are both regarded as moderately resistant but they had quite

different reactions to strain 077-2. Similarly, Doikam is regarded as a susceptible variety but it had a higher level of resistance than most other genotypes to the virulent strain 063-1.

DISCUSSION

Data obtained in this series of experiments supported the conclusions of Jainkittivong *et al.* (1989) and Hokawat and Rudolph (1988) that there were varying levels of virulence in the bacterial pustule pathogen occurring in Thailand and varying levels of resistance among soybean genotypes. If we accept that virulence and resistance are genetically determined then it is highly likely that the inheritance of resistance is complex.

Table 1 Disease severity of bacterial pustule on twelve soybean varieties at twelve locations in Thailand.

Variety	CM[1]	NS	ST	PB	KKU	LO	KPS	SF
SJ 1	2[2]	1	4	3	1	0	4	4
SJ 2	2	2	4	3	1	1	2	2
SJ 4	2	2	4	3	2	0	3	4
SJ 5	2	0	4	3	2	0	1	4
Nakhon Sawan 1	2	0	2	2	0	0	1	2
Sukhothai 1	2	0	1	2	0	0	0	1
AGS 129	2	1	1	2	1	0	0	1
CNS	2	0	1	3	0	0	0	2
AGS 269	1	0	2	2	0	0	0	2
Line 7016	3	1	3	4	3	3	4	4
G 8190	1	0	1	2	0	0	0	1
V 15	2	0	2	3	2	0	3	4

1 Locations: CM Chiang Mai Field Crops Research Centre
NS Nakhon Sawan Field Crops Research Centre
ST Sukhothai Field Crops Experiment Station
PB Phra Phuttabhat Field Crops Experiment Station
KKU Khon Kaen University
LO Loei Field Crops Experiment Station
KPS Kasetsart University Kamphaengsaen Campus
SF Kasetsart University Suwan Farm

2 Details of severity scores are presented in the text.

Table 2 Effect of inoculation times on the disease severity of bacterial pustule on two soybean varieties inoculated by a spray method on whole plants and a wipe method on detached leaves.

Inoculation:	Whole plants		Detached leaves	
Days after sowing	SJ 5	ST 1[1]	SJ 5	ST 1
21	2.7[2]	1.6	1.4	1.1
28	3.6	2.4	2.2	1.1
35	4.6	2.35	3.2	1.5
42	3.4	3.6	2.6	1.6
49	2.75	2.4	3.0	1.4

1 Sukhothai 1

2 Number of pustules per leaf two weeks after inoculation.

Table 3 Effect of growth stage of soybean variety SJ 5 on number of pustules developed following inoculation of either whole plants or detached leaves, which were inoculated by two methods.

Growth stage	Whole plant	Detached leaves	
		Wipe	Pinprick
V 2	3.84	14.57	--
V 3	5.89	21.76	48.25
V 4	9.32	15.80	55.66
V 5	6.78	8.76	36.91
V 6	6.83	18.46	35.91
V 7	9.33	12.23	43.33
V 8	--	--	25.16

Table 4 **Number of pustules per leaflet of six soybean varieties inoculated by the pinprick method with five isolates of the bacterial pustule pathogen (*Xanthomonas campestris* pv. *glycines*).**

Variety	Resistance[1]	014	077-2	063-1	101-1	094-1	Mean
		NS[2]	PB	ST	SF	KKU	
SJ 5	S	25.4	18.3	28.0	0.4	9.4	16.3
Doikam	S	23.7	11.7	9.2	2.6	10.6	11.6
Sukhothai 1	MR	18.1	2.0	10.3	0.0	0.0	6.1
G 8499	MR	29.9	1.9	30.8	3.2	0.0	13.2
Nakhon Sawan 1	R	20.7	22.2	16.4	2.7	6.8	13.8
G 8190	R	20.6	0.4	28.4	1.5	0.0	10.2
Mean		23.1	9.4	20.5	1.7	4.5	11.9

1 From observations in the field. S = susceptible, MR = moderately resistant, R = resistant.
2 Codes for location of collection of strain as for Table 1.

Problems of selection in the field are illustrated by the disease reactions (Table 1) of the four SJ varieties which were all scored 4 at Sukhothai but were scored 1, 2, 3, and 4 at Kamphaengsaen. At Chiang Mai, each of the susceptible SJ varieties was scored 2 which was the same as the relatively resistant varieties Sukhothai 1 and CNS.

Much of the variation in the field was probably due to environmental effects rather than differences in virulence of the pathogen at different locations. Strain 077-2 from Phra Phuttabhat had only low to moderate virulence (Table 4) but this location had the highest mean disease severity score (Table 1). Many years of trials at different locations have shown that high levels of infection are regularly obtained at Phra Phuttabhat and this location is commonly used to screen for resistance. But if the strain from this site is typical of the pathogen population, selection in the field may not have identified lines with resistance to more virulent strains that have been collected from other soybean growing areas.

Variation in the field environment, including variation in the pathogen, reduces the effectiveness of selection in the field for resistance. Use of a reliable controlled screening procedure would overcome this problem. Application of inoculum to plants in a partially controlled environment such as a glasshouse provides a partial solution but the amount of space required to grow large populations, and difficulties in achieving adequate control of temperature and humidity following inoculation, limit the application of this method. An improvement over the inoculation of 'advanced' plants was provided by the technique of Cook *et al.* (1990) in which the cotyledons on one-week-old seedlings were inoculated and resistant genotypes selected within six days.

The detached leaf technique has advantages over other screening procedures. Plants for test can be grown in the field during the dry season or at locations where natural infection is minimal so that large quantities of suitable material can be produced with minimum inputs. Airconditioned laboratory space and simple shelving to support the relatively cheap glass or plastic culture dishes are the only resources required to implement a testing program. (It is assumed that all pathology laboratories would be adequately eguipped to isolate and maintain bacterial pathogen cultures). Since temperature is controlled and humidity is maintained near saturation in the sealed dishes, the environment has minimal effect on disease expression. This level of control also permits a number of different strains of the pathogen to be used in a selection program, or for studies of pathogen variation or the inheritance of disease resistance.

The results indicated that the pinprick method of inoculation is superior to other methods. The grid of pins applies inoculum to a leaf with minimum variability, and is easy and rapid to use. The higher number of lesions that develop with this method indicates its effectiveness. Additionally, lesions develop in about three days compared with two weeks with other methods, allowing the testing of larger numbers of samples. We conclude that this method is the most effective available for screening for resis-

tance to bacterial pustule in soybean and for studies of host-pathogen interactions.

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