Original Research Article

Identification of Resistance Resources to *Xanthomonas axonopodis* pv. *glycines* in Soybean using Excised Leaf Technique

ABSTRACT

Bacterial pustule of soybean is a foliar disease caused by *Xanthomonas axonopodis* pv. *glycines*. The organism produces pustules which causes premature defoliation and ultimately results in significant reduction in seed yield and quality. Use of resistant cultivars can be a cost effective and eco-friendly approach to address the issue. This study was aimed at development of a rapid and reliable *in-vitro* screening method and screening of soybean genotypes for the identification of resistance sources to bacterial pustule disease. One hundred thirty-six soybean genotypes including released varieties, indigenous and exotic germplasm were screened by excised leaf technique for their reaction to bacterial pustule disease. An improved *in-vitro* technique for screening of bacterial pustule disease was developed**.** Rooting and survival studies indicated that soybean leaves excised from 25 days old plants could survive up to 30 days at room temperature in plain water. Inoculation with bacterial suspension of 10^7 to 10^9 colony forming units (cfu/ml) at 27±1°C under the photoperiod of 12h/day light showed initiation of chlorotic lesions 48 hours after inoculation. Based on the latent period and appearance of the chlorotic lesions, the genotypes were classified as susceptible, moderately resistant or resistant. Four soybean genotypes (TS-3, P-4-2, Hara Soya and Himso 1685) were resistant while, seventeen genotypes (AMS MB5-19, Bhatt, DSb-12, JS-335, JS93-05, MACS- 1188, MAUS- 71, PK-262, PS- 1241, Pusa-5, RKS- 18, SL-688, SL-744, SL-958, SL97-52, SL-979, and T99-76) were moderately resistant and rest of the genotypes were susceptible to bacterial pustule disease.

Keywords: bacterial pustule, disease, excised leaf technique, resistance, screening, soybean, Xanthomonas axonopodis pv. glycines.

1. INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is an important grain legume produced throughout the world. It is the leading oil-seed crop produced in the world as well as in India in 2020-21 [1]. Importance of soybean is primarily associated with high quality protein as it contains about 40% protein and 20% oil; and is the richest and cheapest source of most easily accessible vegetable protein [2]. Soybean is used both for human and animal consumption and as well for industrial purposes. Soybean alone accounted 45% of the total oilseed and 25% of the total edible oil produced in the country [3]. The productivity of soybean in India is very low (882 kg/ha) as compared to the world average of 2508kg /ha [4]. Reduction in yield potential

of soybean could be attributed to various biotic and abiotic factors. Among the biotic stresses, bacterial pustule (BP) disease caused by *Xanthomonas axonopodis* pv. *glycines* (*Xag*) is a major bacterial disease of soybean prevalent worldwide [5].
BP is a foliar disease and is characterized by small yellow foliar lesions with raised pustules that ultimately merge into a large necrotic area causing premature defoliation and eventual yield loses [6]. The disease poses a significant concern for soybean producers globally as it causes considerable yield losses and reduces the quality of seeds [5], [7]. In India, BP disease although is distributed throughout the soybean growing regions, is more prevalent in the states of Madhya Pradesh, Rajasthan, Himachal Pradesh, Uttarakhand, and North Eastern States [3], [8]. In India, BP disease incidents ranging from 10 to 80%, and yield losses up to 37% under favorable conditions have been reported [6].
Adoption of appropriate cultural practices and growing disease resistant cultivars are some of the major sustainable, economical and environment friendly approaches for disease management. Varying degrees of resistance to BP disease have been reported in soybean genotypes [9], [10]. Studies have indicated that the BP disease resistant genotypes differ not only in limiting the number of pustules but also in the manifestation of the symptoms [10], [11]. The screening for BP disease in natural field conditions is highly dependent on various factors like inoculum load, growth stage of the plant at infection and presence of congenial environmental conditions etc. The excised leaf technique is an *in-vitro* technique which uses excised leaves from the plants for disease screening [12] and offers a rapid screening of large population without the need for infecting the whole plants.
This study was aimed at the developing a rapid and reliable *in-vitro* screening method and screening of soybean genotypes for the identification of resistance sources to BP disease.

2. MATERIALS AND METHODS

In the present study, 136 soybean genotypes including released varieties, indigenous and exotic germplasm were used for screening. The seeds were obtained from Dr. Punjabrao Deshmukh Krishi Vidyapeeth, Akola; Indian Institute of Soybean Research, Indore and National Bureau of Plant Genetics Resources, Akola. All the soybean genotypes were raised at Experimental Gamma Field Facility, Bhabha Atomic Research Centre, Mumbai during *Kharif* 2019-20, and normal agricultural practices were followed.

2.1 Standardization of excised leaf technique

Fully expanded third trifoliate leaves were excised above the pulvinus from 25, 35 and 45days old soybean plants. Petioles were then inserted through a hole in 2mm thick, opaque plastic sheets and held in position with cotton plugs. The plastic sheets with leaves were placed over enamel tray containing tap water in such a manner that the lower 2cm of the petioles were submerged [12]. In order to study the effect of temperatures, the trays were kept in growth chamber maintained at different temperatures of 22°C, 25°C, 27°C, and 30°C under 12-h/day photo period using white fluorescent light of 4136 lux/m^2 illuminance. The observations on rooting and survival of leaves were recorded until senescence.

2.2 Molecular confirmation of *Xag* pathogen

A *Xag* strain collected from Akola, Maharashtra was maintained in the lab and was used in the study. Four random colonies from LB agar plate were picked up and individually subjected to colony polymerase chain reaction (PCR) based molecular identification of the pathogen. Two pathovar specific primers, viz. heu2 (5'-GACCGAAATGTATTCTTGGG-3') and heu4 (5'-CATTGCGACTAGCAAGG-3') as described by Oh et al. [13], were used for the purpose of specific detection of the pathogen. A colony of *E. coli* was used as negative control. PCR was carried out using a reaction volume of 20µl consisting of 1X PCR reaction

buffer, 2mM $MgCl_2$, 2mM dNTPs, 0.5 U Taq DNA polymerase, 10ng/µl primer (forward and reverse) and a single bacterial colony. Amplification program consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec and a final extension step at 72°C for 7 minutes. Amplifications were performed using a thermal cycler (Eppendorf Master Cycler, Germany). The amplification products were resolved on 2% agarose gel along with 100-bp ladder (gene ruler) for allele sizing, stained with ethidium bromide and analyzed using gel documentation system (Syngene G: Box F3).

2.3 *Xag* inoculum preparation

For the production of *Xag* inoculums, single colony isolates were grown for 16 hours in Luria Bertani (LB) broth (per liter containing tryptone – 10 g, yeast extract – 5g, sodium chloride-10 g, pH 6.8) in an orbital shaker incubator at 28±2 °C, and 150 rpm. Bacterial culture was then centrifuged at 10,000 rpm for 10 minutes, and the pellet was washed twice with sterile distilled water. Bacterial pellet was then resuspended in 0.85% saline and *Xag* inoculum concentration was adjusted to 10^9, 10^7, 10^5 and 10^3 cfu/ml for optimization of inoculum concentration. The inoculums were used to inoculate the excised leaves of the soybean and the leaves were incubated at 22°C, 25°C, 27°C, and 30°C. The initiation of disease was observed by distinct chlorotic lesions developed at the inoculation site.

2.4 BP screening by excised leaf technique

One hundred thirty-six soybean genotypes were screened for their reaction to *Xag* using excised leaf technique. Fully expanded third trifoliate leaves were collected from 25days old soybean plants grown in field. For *Xag* screening, artificial injuries to leaflets were made using blunt end of sterile toothpick, and inoculated with 5 µl of *Xag* bacterial suspension of 10^9 cfu/ml at the injured spots. Leaflets inoculated with 5 µl of sterile distilled water served as control. For each genotype, three set of leaves were used for inoculation. The experiment was carried out in plant growth chamber under controlled conditions of 27±1°C temperature, 80% relative humidity, and 12 h/day illumination with 4136 lux/m^2 white light. Based on the latent period and appearance of the chlorotic lesions; the genotypes were grouped into susceptible, moderately resistant and resistant types. Genotypes were classified as susceptible (developing chlorotic lesion at 48 HAI), moderately resistant (showing chlorotic lesions at 72 HAI), or resistant (genotypes not showing any chlorotic lesions even after 96 HAI).

3. RESULTS AND DISCUSSION

In the present study, rooting and survival of excised leaves was highly affected by age of the leaf and incubation temperature. Initiation of rooting was observed in excised leaves after 9-10 days. However, survival of leaves and rooting was maximum from 25 days old plants and it gradually decreased for 35 and 45 days older plants (Table 1). Temperature also played an important role in excised leaf technique and 100% survival of leaves and rooting was observed from 25 days older plants at 22°C and 25°C. With the increase in temperature, survival and rooting percentage decreased gradually as observed with age of the plant (Table 1). The results of effect of leaf age and temperature on rooting and survival of excised leaves are shown in Table 1. In previous studies, rooting was initiated by the application of plant growth regulators (PGRs) like Indole acetic acid and leaves were also cultured in nutrient solutions [14]. However, application of PGRs has been reported to alter disease reaction [15] and therefore should be avoided while studying host pathogen interactions. In the current study, we used only tap water and profuse rooting was observed in the excised leaves at a temperature of 22°C and 25°C and leaves were healthy even after 30 days of

transfer (Fig. 1). These results indicated that temperatures of 22°C to 25°C and leaf from 25 days old plant were most suitable for excised leaf technique in soybean.

Table 1. Effect of leaf age and incubation temperature on rooting and survival of excised leaves in soybean

Temperature (°C)	Plant age (Days after sowing)	Rooting and survival %		
		10 Days after transfer	20 Days after transfer	30 Days after transfer
22	25	100.0	100.0	100.0
	35	96.4	96.4	96.4
	45	90.0	90.0	90.0
25	25	100.0	100.0	100.0
	35	84.6	92.8	92.8
	45	80.0	80.0	73.6
27	25	85.7	86.4	86.4
	35	80.0	57.1	45.0
	45	82.0	20.7	10.2
30	25	80.0	47.1	30.7
	35	80.0	36.4	23.5
	45	50.0	25.0	17.5

The *Xag* isolate was confirmed by colony PCR using *Xag* specific heu2 and heu4 primer pair. The *Xag* isolate produced a unique 860 bp fragment considered to be specific to *Xag* pathovar (Fig. 2). The use of pathovar specific primers offers fast and sensitive molecular detection of pathogens and has been reported in many studies [13].

Differences in the progression of the disease on the leaves, at different inoculum concentrations and different incubation temperatures are depicted in Table 2. The initiation of chlorotic lesions was observed within 48HAI with high concentration (10^7 to 10^9 cfu/ml) of inoculum at 27°C was clearly discerned at 72HAI. The severity of lesion increased with the increase in incubation period. The inoculum concentration of 10^7 to 10^9cfu/ml was found to be effective in causing disease. The inoculum concentration of 10^8cfu/ml has been found effective on fully expanded trifoliate leaves [6], [11], [16], [17] and on cotyledons [18] by various researchers which is in line with the present study. The optimal temperature for disease development was found to be 27±1º C under 12-h/day illumination with 4136 lux/m^2

of fluorescent white light. Similar results were also reported by cook et al.[18] and Sharma et al. [9]. The injury made on the leaf and deposit of known concentration of inoculum helps to have a uniform disease [19] as was observed in the present studies.

Fig. 1. Excised leaf technique showing the rooting in trifoliate soybean leaves

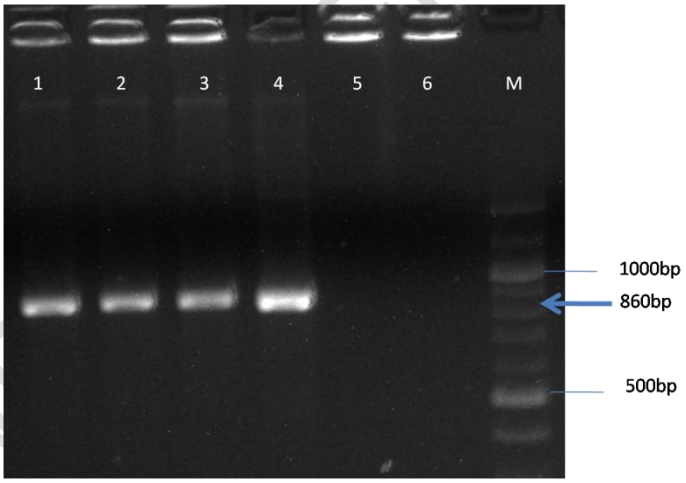

Fig. 2. Ethidium-bromide stained gel of PCR amplification products detected using heu2 and heu4 primers. 1-4, *Xag*, 5-6 *E.coli*, 7- 100bp molecular ladder

Table 2. Response of soybean to different *Xag* inoculum concentration and incubation temperatures

Temperature	Inoculation	Disease symptoms at Hours after inoculation

	concentration	48	72		120
22°C	10^3	-	-	-	-
	10^5	-	-	-	-
	10^7	-	-	-	-
	10^9	-	-	-	-
25°C	10^3	-	-	-	+
	10^5	-	-	-	+
	10^7	-	-	+	+
	10^9	-	-	+	+
27°C	10^3	-	-	-	+
	10^5	-	-	-	+
	10^7	+	+	+	+
	10^9	+	+	+	+
30°C	10^3	-	-	+	+
	10^5	-	-	+	+
	10^7	+	+	+	+
	10^9	+	+	+	+

+ = arbitrary unit of disease severity
- = no symptoms

In the present study, 136 soybean genotypes were screened for BP resistance using excised leaf technique and inoculum concentration of 10^9cfu/ml. Based on the latent period and appearance of the chlorotic lesions, the genotypes were grouped into susceptible (48 HAI), moderately resistant (72HAI) and resistant types (no chlorotic lesions even after 96 HAI) (Fig. 3). In the study, four soybean genotypes (TS-3, P-4-2, Hara Soya and Himso 1685) were identified as resistant, seventeen genotypes (AMS MB5-19, Bhatt, DSb-12, JS-335, JS93-05, MACS- 1188, MAUS- 71, PK-262, PS- 1241, Pusa-5, RKS- 18, SL-688, SL-744, SL-958, SL97-52, SL-979, and T99-76) as moderately resistant and rest of the genotypes were found susceptible to BP disease (Table 3). Earlier, genotypes (P-4-2 and P-169-3) have been reported resistant to BP under artificial conditions [9], [20]. Field screening and identification of resistance to *Xag* has been reported by various researchers [8], [10], [21], [22]. However, genotypes may show different reaction to disease in field and artificial

conditions. For example, soybean cultivar CNS showed high degree of resistance to BP disease under field screening [23]. However, CNS showed susceptible reaction in *in-vitro* under artificial conditions [16]. Similar results have also been reported earlier [9]. To accelerate breeding and selection of resistance sources for various biotic stresses, availability of a rapid and reliable screening method is essential. The *in-vitro* excised leaf method used in this study allowed screening of large number of soybean genotypes for resistance to BP disease in a rapid and reliable manner within a limited space. It is a nondestructive method, wherein plants are in the field and only excised trifoliate leaves are used for screening. The resistant source for BP in all the four resistant genotypes viz. 'P-4-2, TS-3 (JS 80-21 X P-4-2'), Hara Soya (Himso-1520 × Bragg), and Himso 1685 (H 330 x Hardee) were from Palampur, Himachal Pradesh. These genotypes would serve as an important genetic resource for BP disease resistance breeding in soybean improvement programs. Genetic analysis of resistance to BP disease in the soybean genotype P-4-2 showed that the resistance is controlled by two independent recessive genes [20]. The inheritance of BP disease resistance in the resistant genotypes ('TS-3, Hara Soya, Himso 1685') and moderately resistant genotypes ('AMS MB5-19, Bhatt, DSb-12, JS-335, JS93-05, MACS- 1188, MAUS- 71, PK-262, PS- 1241, Pusa-5, RKS- 18, SL-688, SL-744, SL-958, SL97-52, SL-979, and T99-76') also needs to be investigated. Earlier, polymerase chain reaction (PCR) based molecular markers linked to BP disease resistance have been reported in soybean which includes simple sequence repeat (SSR) markers [24], 25], [26], and single nucleotide polymorphism (SNP) markers [26]. However, most of these markers were identified using specific mapping populations and markers were not tightly linked to the resistance gene(s). Therefore, there is a need to identify and validate new markers tightly linked to BP disease resistance. It would be interesting to map resistance gene(s) in the BP resistant and moderately resistant genotypes identified in the present study that would enlighten the nature of the genes imparting resistance and the excised leaf technique would help in rapidly phenotyping the mapping populations for BP disease.

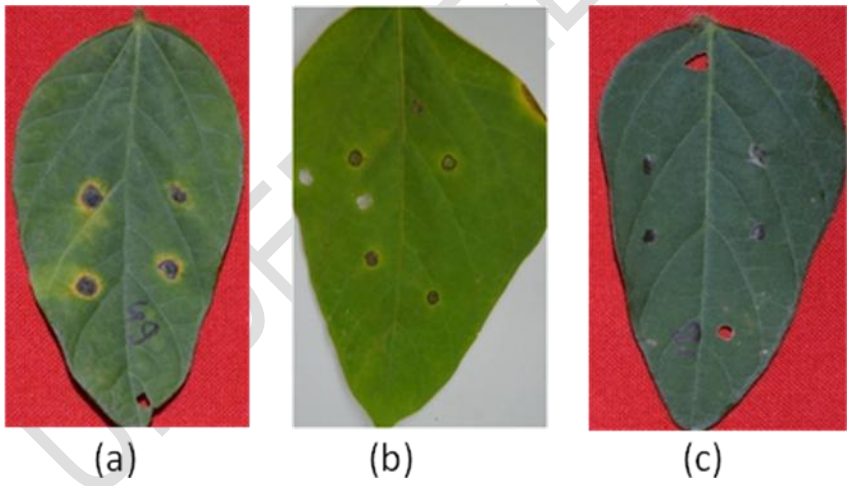

Fig. 3. Induction of chlorotic lesions on soybean by *Xag* inoculum, (a) Susceptible (PK-472); (b) moderately resistant (DSb-12); and (c) resistant (TS-3) genotypes.

Table 3. Reaction of soybean genotypes against *Xag*in laboratory

Susceptible genotypes	ADT-1 (UGM-33), Alankar, Birsa Soya-1, Bragg, Co-1, Co-Soya-2, Co-Soya-3, JS72-280 (Durga), DS-228, DS98-14, DS97-12, Gaurav, Guj Soya-1 (J-231), Guj Soya-2 (J-202), Hardee, Indira Soy 9, Improved Pelican, JS-2, JS71-05, JS75-46, JS76-205, JS79-81, JS80-21, JS90-41, DSb-21, Kalitur, KB-79 (Sneh), KHSb-2, Lee, LSb-1, MACS-13, MACS-57, MACS-58, MACS-124, MACS-450, MAUS-1, MAUS-2 (Pooja), MAUS-32 (Prasad), MAUS-47, MAUS-61 (Pratikar), MAUS-61-2 (Pratishta), MAUS-81 (Shakti), Monetta, NRC-2 (Ahilya-1), NRC-7 (Ahilya-3), NRC-12 (Ahilya-2), NRC-37 (Ahilya-4), Palam Soya, PK-308, MACS-1188, PK-416, PK-471, PK-472, PK-564, PS-1024, PS-1029, PS-1042, PS-1092, PS-1225, PS-1347, Punjab-1, Pusa-16, Pusa-20, Pusa-22, Pusa-24, Pusa-37, Shilajeet, Shivalik, SL-295, SL-525, TAMS-38, RKS-24C, Type-49, VL Soya-1, VL Soya-2, VL Soya-21, VL Soya-47, VL Soya-59, VL Soya-63, DSb-1, Samrat, IC-202, IC-18758, IC-96297, IC-96245, IC-96382, IC-118041, IC-118047, IC-11853, IC-118054, IC-118058, IC-118183, IC-118268, IC-118296, EC-18735, EC-39076, EC-77214, EC-106992, EC-113394, EC-241755, EC-251358, EC-251523, EC-280125, EC-280132, EC-341755, EC-389148, EC-389159, EC-389165, EC-389166, EC-389170, EC-389178, EC-389179, EC-389392, EC-389400, EC-390981, EC-391172, EC-391181, EC- 241780
Moderately resistant genotypes	AMS MB5-19, Bhatt, DSb-12, JS-335, JS93-05, MACS- 1188, MAUS- 71, PK-262, PS- 1241, Pusa-5, RKS- 18, SL-688, SL-744, SL-958, SL97-52, SL-979, T99-76
Resistant genotypes	TS-3, P-4-2, Himso-1685, Hara Soya (Himso 1563)

4. CONCLUSION

An improved laboratory technique of screening for BP disease resistance was developed. Screening of 136 soybean genotypes using excised leaf technique identified four resistant (TS-3, Hara Soya,Himso-1685 and P-4-2) and seventeen moderately resistant (AMS MB5-19, Bhatt, DSb-12, JS-335, JS93-05, MACS- 1188, MAUS- 71, PK-262, PS- 1241, Pusa-5, RKS- 18, SL-688, SL-744,SL-958, SL97-52, SL-979, and T99-76) genotypes. These genotypes would serve as important genetic resources for breeding BP disease resistance and associated molecular studies in soybean.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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