Bangladesh J. Bot. **34**(1): 11-16, 2005 (June)

A SIMPLE AND RELIABLE METHOD FOR PATHOGENICITY TESTS OF BACTERIAL BLIGHT DISEASE OF RICE

MD. ENAMUL HOQUE[*] AND JOHN W. MANSFIELD[1]

Biotechnology Division, Bangladesh Rice Research Institute, Gazipur-1701, Bangladesh

Key words: Xanthomonas oryzae pv. *oryzae, Oryza sativa* L. Pathogenicity tests, Transgenic rice

Abstract

A simple and reliable pathogenicity test for bacterial blight disease of rice was developed under controlled conditions using a small number of rice plants. Between the two bacterial inoculation methods, MII (cutting and pricking) method was found to be better in all the cases irrespective of genotype and incubation period. This method was also found to be effective for resistance scoring in transgenic rice plants containing Xa21 gene.

Introduction

Bacterial blight (BB) of rice, caused by a Gram-negative bacterium, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is the most destructive bacterial disease in Asia and Africa. Recently the disease has also been reported in Australia, USA and several Latin American countries (Ronald 1997). In Bangladesh BB has become a major rice disease in the last few decades since the introduction and widespread growing of nitrogen-responsive modern cultivars. High nitrogen level increased disease and reduced yield in BB-susceptible cultivars. However, BB-resistant cultivars were not affected by the application of high levels of nitrogen, In resistant cultivars, the defense response is characterized by an increase in peroxidase activities, the deponsition of liginin into the plant cell wall, host cell death, and limitation of bacterial multiplication (Reimers *et al.* 1992).

Infection by *Xoo* causes reduction in total dry matter, grain weight of rice, and increase in the number of sterile grains. It also results in poor maturation and broken grain in milling. Generally the extent of damage depends on the severity of the disease and the stage of growth of the rice crop grown at which infection takes place. In the tropics, damage is more severe than in the temperate regions. Ou (1985) reported a loss of 50% yield on very susceptible cultivars and 10% yield on moderately susceptible cultivars, but yield loss in resistant cultivars was insignificant under field conditions. Chemical control of the disease has not been effective or economic. This is why research efforts to control the disease have centered on the development of resistant varieties through conventional breeding methods and also genetic engineering techniques.

Screening for varietal resistance based on natural infection may not always be conclusive due to seasonal variation (environment) and the absence of adequate inoculum that initiates the disease. Artificial inoculation minimizes such problems. Several inoculation techniques have been used to evaluate rice cultivars for resistance to *Xoo* (Mew, 1987). They could be grouped into the needle-pricking method (Ou 1985), clipping method (Kauffman *et al.* 1973), dipping method (Zaragosa and Mew 1979) and spraying method (Mew 1987, Mew *et al.* 1981). Most of these screening techniques require wounding of the host tissues in order to introduce the inoculum to the infection site.

[*]Corresponding author. [1]Department of Agricultural Sciences, Imperial College London, Wye campus, Ashford, Kent, TN25 5AH, UK.

The main objectives of the pathogenicity tests conducted were to establish a suitable method for disease development using single rice plants in controlled conditions applicable to transgenic rice and to test the susceptibility of Bangladeshi rice genotypes to *Xoo* isolate Bxo9.

Materials and Methods

Plant materials: Five rice genotypes were used for pathogenicity tests: four Indica, Bangladesh rice genotypes (Moulata, BR5842-15-4-8, BR22 and BRRI Dhan 29) and the Japonica cultivar Taipei-309 which is susceptible to *Xoo* has been used as control. The *Xa21* gene (confer resistance to *Xoo*) containing transgenic Taipei-309 rice plants were also used in the studies.

Bacterial isolation: One *Xoo* isolate, Bxo9 was used in these studies, which was collected from the Bangladesh Rice Research Institute (BRRI), Gazipur-1701, Bangladesh. The bacterial strain was isolated from *Xoo* infected leaves. King's medium (KB), a semisolid medium was used for bacterial isolation from infected rice leaves (King *et al.* 1954).

Infected leaves were cut into small pieces, which were then surface sterilized by immersing them in 70% (v/v) ethanol for 1 min followed by two washings with distilled water. After that the leaf pieces were soaked in 50 ml sterile distilled water for 30 min at room temperature to allow bacteria to disperse into the surrounding liquid. After 30 min the water became cloudy which indicated the presence of a high number of bacteria. A loopful of the washings was streaked onto KB medium and incubated at 30°C for 48 h. After 48 h several yellowish watery colonies appeared on the plate. Further streaking on KB Petri plates allowed single colonies and pure cultures to be obtained.

Growth and maintenance of the bacterial culture: Bacterial isolates were routinely grown on Luria-Bertani (LB) agar or LB broth at 25 - 28°C. Liquid cultures were shaken at 150-200 rpm in an orbital incubator (Gallenkamp). Cultures for routine use were stored at 4°C. For long-term storage, cultures were maintained in glycerol (15% final concentration) at -70°C.

Plant germination and growth for pathogenicity tests: Rice seeds were soaked in water and kept overnight at 30°C. Then the seeds were placed on moist filter paper at 30°C for 3/4 days. Germinated seeds were then sown in 15 cm diameter plastic pots containing John hIDes No.2 loam-based compost (J. Arthur Bower's, UK). Pots with plants were kept in a growth cabinet maintained under 12 h photoperiod with a mean day : night temperature of 25 : 20°C and a relative humidity of 80%. Six to eight week old rice plants were used for the pathogenicity test.

Pathogenicity tests: The bacterial culture was grown overnight at 25°C in 10 ml LB broth on an orbital shaker at 200 rpm. The resulting bacterial suspension was centrifuged at 3500 g in a bench top centrifuge for 5 min. The supernatant was discarded; bacterial pellet was resuspended in sterile 10 mM $MgCl_2$ solution and recentrifuged as described above. The washed pellet was again resuspended in 10 mM $MgCl_2$ solution and adjusted to a final concentration of 10^9 cfu/ml using a spectrophotometer. Inoculum was tested in five leaves of each genotype examined. Leaf lesion length was measured from the cut surface at the tip to the distant-most position on the leaf that exhibited a gray, chlorotic or water-soaked lesion. Data were taken from five leaves of a plant of each genotype at five-day intervals for up to 15 days after inoculation.

Inoculation method: Leaves were inoculated in two ways: either cutting and dipping (MI), or cutting and pricking (MIl) method.

Cutting and dipping inoculation method (MI): Leaves were inoculated by cutting the leaf 2-3 cm from the tip and dipping the cut end into the bacterial suspension for 1 min. The bacterial suspension was replaced with sterile 10m M $MgCl_2$ in case of control. Inoculated plants were then kept in a plant growth chamber (Fitotron, SANYO Gallenkamp PLC), maintained under a mean day: night temperature of 27: 22°C and 85% humidity with a 12 h photoperiod.

Cutting and pricking inoculation method (MIl): Leaves were first cut 2 - 3 cm from the tip and then inoculated by pressing the cut end of the leaves with rats-toothed forceps that had been dipped into the bacterial suspension. This was done two to three times for each leaf and plants were then maintained as in MI method. Inoculation and resistance scoring of transgenic Taipei-309 plants: Three transgenic plants were randomly selected for resistance scoring and five I eaves were inoculated with the bacterial isolate using the MIl inoculation method. At the same time, 6 weeks old non-transgenic Taipei-309 plants were also inoculated as controls. Plants were then maintained in a Fitotron as mentioned in MI method. Data were taken as described in MI method.

Experimental design: The experiment was done using five leaves for each genotype with each bacterial inoculation method in a completely randomized design. Each leaf was considered as a replicate of each treatment. Due to repeated measurement of lesion length, three analyses of variance (ANOVA) were carried out using the data recorded at 5, 10 and 15-days after inoculation. Means and standard errors of means (SE) were also calculated.

Results and Discussion

Three to four days after inoculation, pale yellowish discoloration was found at the cut end of the leaves of all the plants tested. This 'yellow lesion' gradually expanded with time. Figs. l and 2 show the mean lesion length recorded on five *Xoo* inoculated rice leaves using the two different

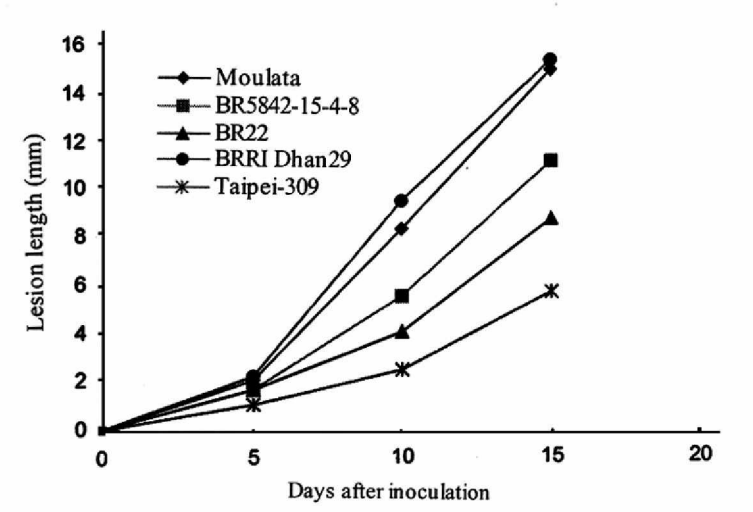

Fig. 1. Symptom development in different rice genotypes after inoculation with the Box9 isolate in MI inoculation method.

inoculation methods in all genotypes. MII caused the almost rapid lesion formation. Statistical analysis (Table 1) showed significant difference between genotypes (G) and the two inoculation methods (M). The G x M interaction was also significant at 5 days after inoculation. After 10

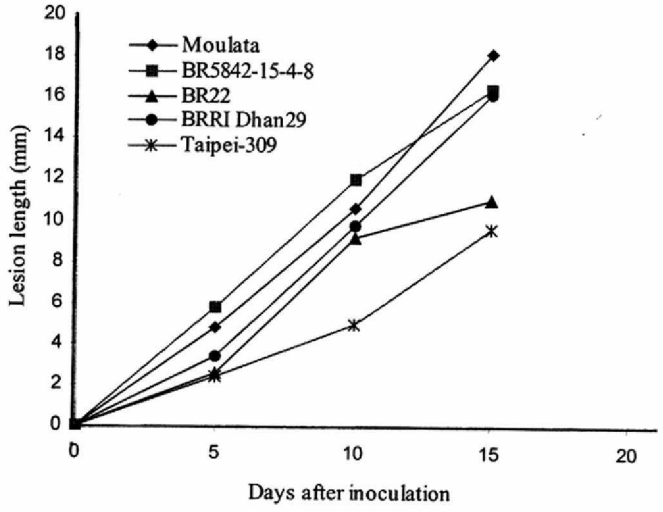

Fig. 2. Symptom development in different rice gentoypes after inoculation with the Box9 isolate in MII inoculation method.

days, genotype (G) and method (M) caused significant difference and after 15 days only the genotypes (G) were significantly different (Table 1). The MII method of inoculation was found to be better in all the cases irrespective of genotype and incubation period. However, lesion length varied depending upon the rice genotype used. Kauffman and Rao (1972) have also reported differential interactions between the isolates and the cultivars even without major gene resistance. In the MIl inoculation method, leaf tips were not only cut but also pressed by the serrated part of a

Table 1. Mean lesion length after inoculation of leaves with the Box9 isolate in five rice genotypes using two different methods.

Genotype	Lesion length (mm) ± SE					
	5-days		10-days		15-days	
	MI	MIl	MI	MIl	MI	MIl
Moulata	2.0 ± 0.32	4.8 ± 0.58	8.2 :t 1.2	10.6 ± 0.87	14.8 ± 0.66	18.4 ± 3.5
BR5842-15-4-8	1.6 ± 0.4	5.2 ± 0.37	5.4 :t 1.17	12.0 ± 2.0	11.0 ± 2.32	16.4 ± 2.73
BR22	1.6 ± 0.25	2.6 ± 0.25	4.0 :t 0.55	9.2 ± 1.59	8.6 ± 0.51	11.0 ± 1.64
BRRI Dhan 29	2.2 ± 0.2	3.4 ± 0.51	9.4 I 1.75	9.8 ± 0.8	15.2 ± 1.77	16.2 ± 2.91
Taipei-309	1.0 ± 0.0	2.4 ± 0.51	2.4 :t 0.25	5.0 ± 0.32	5.6 ± 0.4	9.6 ± 1.57
Two-way table	P (genotype) = < 0.001		P (genotype) = < 0.001		P (genotype) = < 0.001	
	P (method) = < 0.001		P (method) = < 0.001		P (method) = 0.016	
	P (genotype x method)= 0.004		P (genotype x method) = 0.097		P (genotype x method) = 0.86	
	LSD (GxM) = 1.077, df=40		LSD (GxM)= 3.413, df=40		LSD (GxM) = 5.908, df=40	

MI = Cutting and dipping inoculation method, MIl = Cutting and pricking inoculation method. G = Genotype, M = Method

forceps and that might help the bacterium to enter easily into the vascular system of the leaf. All leaves exhibited longer lesions using the MIl inoculation method. With both the MI and MIl methods, all four Bangladeshi rice genotypes showed more susceptibility to the Bxo9 isolate than the Japonica cultivar Taipei-309.

Disease reaction of transgenic Taipei-309 plants: Mean lesion lengths recorded in transgenic and non-transgenic plants are presented in Fig. 3. Lesion length was found to be significantly less in transgenic *Xa21* plants than non-transgenic plants (Table 2, Fig. 3). There was also a significant difference between days, and the interaction between plant type and day.

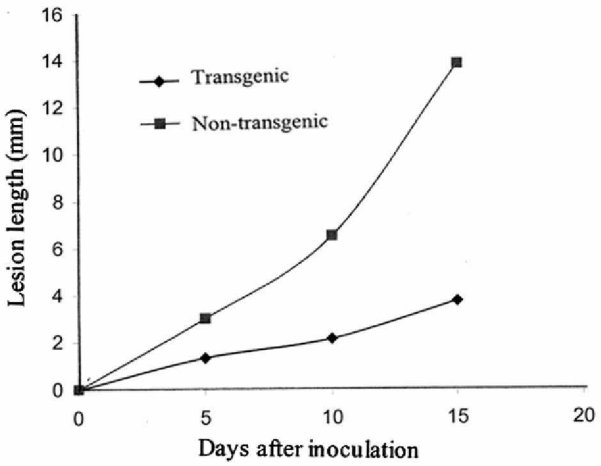

Fig. 3. Symptom development in transgenic *Xa21* and non-transgenic Taipei-309 plants after inoculation with the Box9 isolate in MII inoculation method.

Table 2. Disease reactions of transgenic Taipei-309 plants after inoculation with the Box9 isolate in MIl inoculation method.

Days after inoculation	Mean lesion length (mm) ± SE	
	Transgenic plants	N on-transgenic plants
5	1.3 ± 0.13	3.0 ± 0.17
10	2.1 ± 0.22	6.5 ± 0.22
15	3.7 ± 0.18	13.8 ± 0.41
Two way Table	P (plant type) = < 0.001, P (days) = < 0.001 P (plant type x days) = < 0.001 LSD (plant type x days) = 0.66, df= 84	

In the present study, transgenic Taipei-309 plants containing the *Xa2l* gene were found to be resistant to *Xoo* isolate Box9. Uses of the *Xa2l* gene to confer resistance to different *Xoo* isolates has been reported previously, but not against the Box9 (Tu *et al.* 1998, Wang *et al.* 1996, Zhang *et al.* 1998). Wang *et al.* (1996) tested progeny for resistance to 32 *Xoo* isolates from eight different countries. Both the engineered and donor line showed different degrees of resistance to 29 isolates and susceptibility to three isolates. The identical resistance spectrum of both lines indicated that the presence of a single member of a multigene family, *Xa2l*, is sufficient to confer multi-isolate

resistance. Therefore, the introduction of the *Xa2l* gene into the highly susceptible Bangladeshi rice genotype remains a high priority.

In conclusion it can be said that the cutting and pricking (MII) inoculation method proved to be a simple and reliable method for pathogenicity tests against *Xoo* isolate Box9, which may be applicable to disease scoring in transgenic rice plants.

References

Kauffman, H.E. and P.S. Rao. 1972. Resistance to bacterial leaf blight in India. *In:* Rice Breeding. pp. 283-287. IRRI, Los Banos, Philippines.

Kauffman, H.E., A.P.K. Reddy, S.P.Y. Hsieh and S.D. Merca. 1973. An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. Plant Disease Rep. **57**: 537-541.

King, E.O., M.K. Ward and D.E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Med. **44**: 301-307.

Mew, T.W. 1987. Current status and future prospects of research on bacterial blight of rice. Ann. Rev. Phytopathol. **25**: 359-382.

Mew, T.W., C.M. Vera-Cruz and R.C. Reyes. 1981. Characterization of resistance in rice to bacterial blight. Ann. Phytopathol. Soc. Jap. **47**: 58-67.

Ou, S.H. 1985. Rice Disease, 2nd edn. pp. 61-96. CMI, Kew, England.

Reimers, P.J., A. Guo and J.E. Leach. 1992. Increased activity of a cationic peroxidase associated with an incompatible interaction between *Xanthomonas oryzae* pv. *oryzae* and rice (*Oryzae sativa*). Plant Physiol. **99**: 1044-1050.

Ronald, P.C. 1997. The molecular basis of disease resistance in rice. Plant Mol. Biol. **35**: 179-186.

Tu, J., I. Ona, Q. Zhang, T.W. Mew, G.S. Khush and S.K. Datta. 1998. Transgenic rice variety 'IR72' with *Xa21* is resistant to bacterial blight. Theor. Appl. Genet. **97**: 31-36.

Wang, G.L., W.Y. Song, D.L. Ruan, S. Sideris and P.C. Ronald. 1996. The cloned gene, *Xa21*, confers resistance to multiple *Xanthomonas oryzae* pv. *oryzae* isolates in transgenic plants. Mol. Plant-Microbe Interact. **9**: 850-855.

Zaragosa, B.A. and T.W. Mew. 1979. Relationship of root injury to the "Kresek" phase of bacterial leaf blight of rice. Plant Dis. Rep. **63**: 1007-1011.

Zhang, S., W.Y. Song, L. Chen, D.L. Ruan, N. Taylor, P.c. Ronald, R. Beachy and C. Fauquet. 1998. Transgenic elite Indica rice varieties, resistance to *Xanthomonas oryzae* pv. *oryzae*. Mol. Breed. **4**: 551-558.

(Manuscript received on 6 April, 2005; revised on 12 May, 2005)