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COMPARISON OF METHODS OF INOCULATION OF *XANTHOMONAS ORYZAE* PV.*ORYZAE* IN RICE CULTIVARS

M.A. AKHTAR[1], ABDUL RAFI[2] AND ABDUL HAMEED[3]

[1]*Crop Diseases Research Programme, Institute of Plant and Environment Protection, National Agricultural Research Centre, Islamabad, Pakistan*
[2]*Department of Plant Pathology, NWFP Agricultural University, Peshawar, Pakistan*
[3]*Department of Biological Sciences, Quaid-e-Azam University, Islamabad, Pakistan.*

Abstract

Bacterial suspension containing 10^8 colony forming units (cfu) of *Xanthomonas oryzae* pv. *oryzae* was used for inoculating detached leaves of 6 commercial rice cultivars *In vitro* as well as for testing these cultivars in pots in growth chamber conditions by three methods of inoculation i.e., clipping, pin prick and brush respectively. Clipping method resulted in much more lesion length development than either the pin prick and brush method.

Introduction

Rice (*Oryza sativa*) is one of the most important food crops in the world, feeding about half of humanity. It is the second major cereal crop of Pakistan after wheat. Rice is grown over 2.6 million ha with a production of 5.6 million tons and stands third in terms of area under cultivation (Anon., 2005-06). It is an important part of the diet of the people of Pakistan and is also a valuable source of foreign exchange earnings for the country.

During the year 2005-06, US $ 1158 Million worth of rice was exported to other countries (Anon., 2007). Pakistan's 'Super Basmati' and 'Kernal' rice are world famous for their taste and invigorating aroma. It is, however, unfortunate that such an important crop is attacked by considerable number of diseases, of which bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (Ishiyama, 1922) Swings is one of the most destructive diseases throughout the world (Mew, 1987). This disease is said to have been observed first by farmers in Japan during 1884-85. Its occurrence has been reported from Australia, Bangladesh, Cambodia, Indonesia, India, Korea, Mainland China, Malaysia, Srilanka, Thailand, Philippines, USA, West Africa and Vietnam (Ezuka & Kaku 2000). Mew & Majid (1977) reported the disease for the first time in Pakistan and its occurrence was confirmed from all the provinces in a later study (Akhtar and Akram, 1987). It has been observed during recent years that BLB incidence is increasing in Pakistan especially in "Kaller" belt which is famous for rice cultivation (Khan *et al*., 2000).

BLB occurs at all the growth stages of rice and is manifested by either leaf blight or "kresek" symptoms. The causal organism invades plants through water pores and wounds (Mizukami, 1956, Tabei & Mukoo, 1960). Since the water pores are located at the margins of upper parts of the leaf, the lesion usually starts from the leaf margin near its tip. As the disease progresses, the tiny water soaked lesion turns yellow, enlarges in size progressively and develops into an elongated irregular lesion with wavy margins. Bacterial ooze, which consists of small, yellowish, spherical masses, may sometimes be seen on the margins or veins of the freshly infected leaf under moist conditions. With the passage of time, the lesion may cover on the entire blade, which turns white and later grayish owing to saprophytic growth (Tagami & Mizukami, 1962; Octa, 1970; Ou, 1985). If plant ever produces panicles, it results in sterile immature grains, which are easily

broken during milling. There may be 50% reduction in yield in case of severe infection (Mew *et al*., 1993) whereas 10-12% yield reduction has been recorded in case of mild infection (Ou, 1985).

The disease is also characterized by a systemic infection phase, which is manifested by acute wilting of young plants. This is commonly referred to as the "kresek" phase (Reitsma & Schure, 1950). The causal organism consists of straight rods, with a single polar flagellum, occurs singularly or in pairs or sometimes in chains and is Gram negative (Swings *et al*., 1990). The bacterium over winter either in perennial weeds or in soil, grains, straw and rice stubble are other possible sites of over wintering of the pathogen. During growing season, it enters the plants *via* natural openings or wounds where it survives and multiplies in plant's vascular system, producing typical leaf blight symptoms.

Bacterial blight has the potential to become a destructive bacterial disease of rice in Pakistan and can cause huge losses mainly because information regarding the pathogen and its effective control measures is lacking. Resistance to BLB is known to be widely different with rice cultivars. This is due to the fact that the presences of different pathogenic races subsequently break the resistance of rice cultivars. So evaluating rice cultivars for BLB resistance is a routine practice to overcome yield losses. The purpose of the present study was, therefore, to develop a simple, quick and reliable method of inoculation of different rice cultivars with *Xanthomonas oryzae* pv. *oryzae*.

Material and Methods

Isolation of causal bacterium: The rice leaves affected with bacterial leaf blight were collected from rice field of Punjab, during 2006. Diseased leaf samples were cut into small pieces, 1 cm in length including the marginal portion of fresh lesions. These were placed in 70% Ethyl alcohol for a few seconds, dipped in 1% sodium hypochlorite solution for 1 minute and rinsed in sterile distilled water thrice. Each sample was then homogenized with 10 ml sterile distilled water. The resulting suspension was diluted with sterile distilled water and appropriate dilution was thoroughly mixed with melted nutrient agar medium kept at 50^{o}C in a water bath. The mixture was poured into a plate and the plates were incubated at 25^{o}C for 4 days. Single-colony isolation was made. The viscous and yellow bacterial colonies that subsequently developed were subcultured on peptone sucrose agar (PSA) medium and grown at 25^{o}C for 2 days (Devadath, 1989). For long-term preservation, the bacterial cells suspended in 10% (w/v) skim-milk containing 0.05% L-glutamic acid were stored at 0^{o}C until needed.

Inoculation: Six commercial rice cultivars viz., BAS 2000, BAS 385, JP5, IR6, BAS 370 and Super BAS, were used in the experiment. Plastic pots (10 cm diam) containing green house potting mixture, were planted with 10 seeds per pot. A culture of *X.o* pv. *oryzae* was revived on yeast extract dextrose Calcium corbonate (YDC) (Schaad, 1980) at 27^{o}C for 72h., for inoculation, bacterial concentration in 0.01m phosphate buffer pH 7.2 was ascertained with a spectronis 20 (Bush & Lomb) at 590 nm and adjusted to 10^{8} colony forming units (cfu)/ml). The inoculation methods were as follows:

Detached leaf assay: The leaves were thoroughly washed under running tap water to remove dirt and then surface disinfected in 70% Ethanol for I minute and rinsed thrice with sterile distilled water. The leaves were placed abaxial side upward on four layers of sterile paper towel on two glass slides in 90 mm Petri plate. The leaves were then

inoculated with a multiple pin mount prepared with 4 insect mounting pins of 0.1 mm diameter in a 25 mm square piece of Styrofoam. The pins were dipped in the bacterial suspension of strain in 0.1 M Phosphate Buffer Saline (PBS) containing 10^8 cfu/ml as determined with a spectronic 20 (Baush & Limb) adjusted to an absorbance of 0.1 at 590 nm. The leaves were then pricked with these pins. After inoculation three leaves were kept in each Petri dish. Similarly the leaves were inoculated by dipping the inoculating scissor in inoculum kept in a beaker and the third method the inocolum was applied with brush. Each treatment was replicated three times. In control treatment leaves were inoculated with PBS only. The leaves were incubated at 27^0C under 14 hr illumination. Disease data were recorded on lesions length in cm after 14 days of inoculation.

Pot experiment: Six commercial rice cultivars viz., BAS 2000, BAS385, JP5, IR6, BAS 370 and Super BAS were used in the experiment. Plastic pots (10 cm diam) containing green house potting mixture were planted with 10 seeds per pot. Three leaves/ plant and three plant in each pot were inoculated with the bacterial suspension of 10^8 cfu/ml prepared in PBS. Leaves of the rice seedling were moistened with serile water and bacterial suspension was then applied on the leaves using three methods of inoculation as previously described. The leaves of control treatment were inoculated with sterile PBS only. After inoculation the leaves were covered with polyethylene bags containing water saturated cotton for 24 hr. The plants were kept at 27^0C with 70% relative humidity during study.

Result and Discussion

Six varieties of rice were tested using detached leaves and pot experiments with three methods of inoculations *In vitro* .One month old plants were inoculated with inoculum of bacterial suspension of *X.o* pv. *oryzae.* Two weeks after inoculation, yellow lesion with wavy margin appeared on leaf margin and the leave became yellowish and dry. The data on lesion development was recorded 15 days after inoculation. *X. o* pv. *oryzae* isolate gave differential response with respect to cultivars and method of inoculation. Bas 2000, Bas 385, Super Basmati and Bas 370 were susceptible to Rice bacterial blight disease (Table 1 & 2). Rice varieties Super Basmati, Bas 370, Bas 385, Bas 2000 showed typical disease symptoms with clipping method and exhibited medium susceptibility, but no variety was found resistant. The clipping inoculation method showed typical yellow lesions with wavy margin 2 weeks after inoculation on cultivars Basmati 2000, Basmati 385 and Super Basmati. Based on lesion length scoring the susceptible and resistant varieties were assessed. Variation in lesion development was noticed with method of inoculation and with respect to cultivar. However clipping method remained the best as compared to brush and pin prick. These results corroborated with Kauffman *et al*., (1973). The brush and pin pricking method expressed less BLB symptom as compared to clipping method. Brush method is least effective as bacteria cannot penetrate the intact host surface. In screening for resistance such method can therefore cannot be used with confidence. Lesion development is significantly effective with method and variety. Moreover on detached leaves lesions appeared within 5 days after inoculation with clipping method in contrast to other methods which took about 15 days on intact leaves. This might be due to a loss of resistance due to excision shocking of incubation period. These studies suggested that clipping inoculation method provides a reliable method for screening of germ plasm resistance against bacterial blight.

Table 1. The effect of different method of inoculation on development of rice blight lesion on detached leaves of rice.

		Rice bight lesion (cm)				
S. No.	**Varieties**	**Clipping method**	**Pin prick method**	**Paint brush method**	**Mean**	**Reaction**
1.	BAS2000	16.9.	15.5	14.8	15.73 cm	S
2.	BAS385	13.5	10.5	9.7	11.23 cm	MS
3.	JP5	10.00	7.00	4.9	7.30 cm	MR
4.	IR6	9.00	5.8	3.3	6.03 cm	MR
5.	BAS 370	17.9	16.5	15.5	16.63 cm	S
6.	Super BAS	17.6	15.5	12.7	15.27 cm	S
Mean		**14.15 cm**	**11.80 cm**	**10.15 cm**		

Comparison of the methods of inoculation on rice varieties against X.oryzae pv.oryzae R: (1---5) resistant with browning margion on around lesions, MR: Moderately resistant (5---10cm), MS: Moderately susceptible (10---15), S: Susceptible (15cm to above)

Table 2. The effect of different method of inoculation on development of rice blight lesion on potted plants leave of rice.

		Rice blight lesion (cm)				
S.No.	**Varieties**	**Clipping**	**Brushing**	**Pin prick**	**Mean**	**Reaction**
1.	BAS2000	17 cm	13cm	15cm	15 cm	S
2.	BAS385	15 cm	8cm	13cm	12 cm	MS
3.	JP5	8 cm	3cm	6cm	5.67 cm	MR
4.	IR6	11 cm	6cm	9cm	8.67 cm	MR
5.	BAS370	17 cm	13cm	15cm	15 cm	S
6.	Super BAS	18.9 cm	16.5 cm	15.8cm	17.07 cm	S
Mean		**14.48 cm**	**9.92 cm**	**12.30 cm**		

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