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Determination of antibacterial activity of various broad spectrum antibiotics against *Xanthomonas oryzae* pv. *oryzae*, a cause of bacterial leaf blight of rice

Syed Atif Hasan Naqvi, Rashida Perveen, Ummad ud Din Umer, Owais Malik, Ateeq ur Rehman, Sajid Wazeer, Taha Majid

Department of Plant Pathology, Faculty of Agricultural Sciences and technology, Bahauddin Zakariya University, Multan, Pakistan

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Abstract

Bacterial leaf blight (BLB) of rice (*Oryza sativa* L.) caused by *Xanthomonas oryzae* pv. *oryzae*, is arguably the most holistic pathosystem of rice throughout the worldwide due to its growing concern as this disease is wide spread, devastating and its control measures are still not well understood. *In vitro* evaluation of various broad spectrum antibiotics viz., streptomycin sulphate, kanamycin sulphate, chloramphenicol, ampicilin trihydrate and benzylpenicillin, was carried out to determine the best chemistry against the destructive pathogen *Xanthomonas oryzae* pv. *oryzae* at different concentrations. Inhibition zones appeared on petri plates for the growth of bacteria were very clear around the paper disks. Chloramphenicol proved to be the most effective antibiotic to control the bacterium as it suppressed the bacterial growth to greater extent and only the 6.25 mean bacterial colonies were appeared in the petri plates, followed by the ampicillin trihydrate which showed to be the second most effective antibiotic against the pathogen growth and retarded to 12.00 mean bacterial colonies. The maximum diameter of inhibition zone (28.31 mm) was showed by the Chloramphenicol at 100 ppm followed by ampicillin trihydrate which gave proved to be second most effective antibiotic to control the pathogen and gave maximum inhibition zone (25.02 mm) at 100 ppm concentration. All the antibiotics showed significant results at higher concentrations. The study suggests that the experiments in the field must be conducted to prove the effectiveness of these broad spectrum antibiotics in the natural environmental conditions as there is a possibility of some variation in the field results because of various factors which influence the chemical management of plant diseases in the field.

* Corresponding Author: Syed Atif Hasan Naqvi ✉ atifhasanshah@hotmail.com

Introduction

Rice (*Oryza sativa*) is one of the most important fruit crop and life for thousands of millions people of the world and nearly half of the population of world directly depends upon this staple food crop for their daily requirements. Cultivation of rice is one of principal practice for earnings of millions of people throughout the world while Asia and Africa are totally depend on rice crop as a big source for earning foreign exchange and government revenue. Pakistan is not among the top rice producing or consuming countries of the world yet it is fifth largest rice exporter in the world (Anonymous, 2009a). Rice is the second largest cereal food crop of Pakistan after the wheat which is the staple food for the people of country. In the export of agricultural food commodities, rice comes second after cotton in Pakistan (Anonymous, 2009b). Pakistan's average yield potential per unit area of rice is too below from the world average production and very low from many neighboring countries (Anonymous, 2009a).

The absence of potential resistance/ tolerance in well known basmati cultivars against biotic diseases which are cultivated mostly in Pakistan is one of the important reasons for the low production of crop (Khan *et al*., 2008). Rice crop in Pakistan is vulnerable to many diseases, out of those holistic diseases bacterial leaf blight of rice generally called (BLB) caused by the gram negative rod bacterium *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Swings *et al*., (1990) is one of the most important and destructive disease of rice crop which is widespread in Asia and throughout the world, particularly this disease is reportedly present in South East Asia, especially in Philippines, Japan, India and Indonesia (Srivastava, 1972; Ahmed & Singh, 1975; Reddy, 1989, Singh *et al*., 1987; Ou, 1985;). Bacterial leaf blight of rice was first time recorded in Pakistan in rice research institute Kala Shah Kaku and the neighboring farmer's fields (Mew and Majid, 1977), and now it is increasing day by day in Pakistan in the recent years particularly in Kaller belt which is very famous for the producing of high quality rice.

Surveys for disease severity in rice growing areas of Punjab during 1997and 1998 showed the presence of bacterial leaf blight of rice in the northern areas of the country (Khan *et al*., 2000a). Akhtar *et al.* (2007) reported that bacterial leaf blight of rice is consistently present in all the provinces of Pakistan and the maximum incidence of disease was noticed in Punjab. Khan *et al.*, (2000b) described epidemics of bacterial leaf blight of rice in some areas of northern and central Punjab during 2006 and 2007. The only economical way out for the control of disease is the use of highly resistant varieties and the avoidance of monocropping practices. Although the fungicides, pesticides, nematicides and bactericides are no doubt among the quickest solution for the sudden outbreaks of diseases and insects pests and their use in the field persuade the farmers for the quick and noticeable actions (Sehgal *et al*., 2001).

Use of antibiotics and even the use of organic compounds such as cow dung were successfully evaluated for the control of bacterial leaf blight of rice (Singh *et al*., 1980; Grainge *et al*., 1985; Mariappan *et al*., 1990; Mary *et al*., 1986; Sreekumar and Nasir, 1990). Although the use of antibiotics is common for the control of bacterial disease yet there is no truly effective bactericide is available for the proper management tactics of the disease and to avoid the epidemics of the disease (Noda *et al*., 1996; Lee *et al*., 2003). Keeping in view the above mentioned status of bacterial leaf blight of rice control tactics, taking into account the magnitude of disease and its unbearable losses, the current study was performed with the firm attitude to evaluate the most efficient antibiotic commercially available against *Xanthomonas oryzae* pv. *oryzae*.

Materials and Methods

Study site

The study was carried out in Mycology and Bacteriology laboratory of Department of Plant Pathology, Faculty of Agricultural Sciences and Technology, Bahuddin Zakariya University, Multan (30° 11′ 52″ N, 71° 28′ 11″ E, 410 ft elevation above sea level) in January 2014.

Sample collection

Disease samples showing the characteristic symptoms of the bacterial leaf blight disease of rice were collected at the Agriculture Experimental Fram, Bahauddin Zakariya University, Multan in from the rice crop, kept in plastic bags and were brought to the Laboratory for further processing.

Pathogen isolation

Excised diseased tissues (0.5-1.0 cm) were disinfected in 1.00 % sodium hypochlorite solution, dried on blotting paper and positioned into autoclaved petri plates lined with Nutrient Agar (NA) (Bio Basic Inc.) at ± 30°C temperature for 72 hr. The isolated bacterial culture was purified again in nutrient broth for shorter period of time.

In vitro evaluation of antibiotics

Bacterial suspension

Standard bacterial suspension was prepared by using the serial dilution technique, taking a loopful of inoculum in the into a flask containing the distilled water which served as stock solution and further dilutions were made by the serial dilution and 10^5 cfu/ml dilution was used for this experiment so that the bacterial colonies might be counted easily and the inhibition zone could be measured with greater accuracy.

Antibiotics and standard concentrations

In the current study five commonly available antibiotics viz., Streptomycin sulphate, Kanamycin sulphate, Chloramphenicol, Ampicillin and Benzylpencillin were evaluated against the *Xanthomonas oryzae* pv. *oryzae*. A stock solution in parts per million (ppm concentration) of each antibiotic was made using (w/v, 1µg/ml) and further four different concentrations viz., 25, 50, 75 and 100 ppm were made by using the stock solution to examine the effectiveness of antibiotics against the colonial growth and inhibition zone of the pathogen.

Disk diffusion technique

Disk diffusion technique can be described as when a filter paper disc impregnated with a chemical is placed on agar and the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "zone of inhibition".

Disk diffusion method by Beuer and Kirby (1985) was used to determine the effectiveness of the antibiotics against the *Xanthomonas oryzae* pv. oryzae. Petri dishes containing only nutrient agar with disks without any antibiotic served as control. Sterilize disks were prepared by the use of hand punch and impregnated on the nutrient agar with the help of sterilized scalpel. 50 µl of each antibiotic was immersed on the disk and bacterial suspension was incorporated on the nutrient agar with the sterilized cotton swab and the petri plates were completely randomized at ± 28 ºC for 72 hrs. Data was collected for the zone of inhibition measured and the number of colonies of bacteria developed on the nutrient agar.

Experimental Design

The laboratory experiment was performed in controlled conditions with completely randomized design (CRD) using the four replicates of each treatment. The standard error was also calculated

to know the variation among the treated petri plates.

Statistical analysis

Data regarding number of colonies of the bacteria grown on the nutrient agar and diameter of the inhibition zone was analyzed statistically by the analysis of variance (ANOVA) and treatment means were subjected to least significant difference (LSD) test at ($P \leq 0.05$) using SAS (Statistical Analysis System, version 9.1).

Results

Bacterial colonies

Various broad spectrum bactericides with different concentrations were evaluated under in vitro conditions and the sensitivity of *Xanthomonas oryzae* pv. *oryzae* was observed, no of colonies of bacteria and diameter of zone of inhibition was recorded. Inhibition zones for the growth of bacteria were appeared to be very clear around the paper disks. Chloramphenicol was found to be the most effective antibiotic against the bacterium as it potentially reduced the bacterial growth and only the 6.25 mean bacterial colonies were appered followed by the ampicillin trihydrate which showed to be the second most effective antibiotic against the pathogen growth and retarded to 12.00 mean bacterial colonies. Streptomycin sulphate and benzylpenicilin were statistically at par and showed mean bacterial colonies to 15.25 and 15.75 respectively. Kanamycin sulphate was the least effective antibiotic against the bacterial growth and showed maximum number of colonies of bacteria to be recovered (Table 1).

Table 1. Mean bacterial colonies grown in petri plates under the effect of disk diffusion technique impregnated with different ppm concentration of antibiotics.

	Colonies of bacteria (Number ± S.E*)				
Concentartion ppm*	Streptomycin Sulphate	Kanamycin Sulphate	Chloramphenicol	Ampicilin trihydrate	Benzylpenicillin
25	15.25 ± 0.85 b	19.00 ± 0.91 b	6.25 ± 0.25 b	12.00 ± 0.41 b	15.75 ± 0.48 b
50	12.25 ± 0.25 c	17.00 ± 1.08 c	5.25 ± 0.25 c	8.50 ± 0.50 c	14.25 ± 0.48 c
75	6.50 ± 0.29 d	12.75 ± 0.63 d	0.00 ± 0.00 d	5.75 ± 0.63 d	10.00 ± 0.41d
100	2.00 ± 0.41 e	11.25 ± 0.25 d	0.00 ± 0.00 d	0.50 ± 0.29 e	8.50 ± 0.29 d
Control	48.25 ± 2.14 a	58.25 ± 2.75 a	48.50 ± 2.22 a	52.50 ± 1.26 a	54.50 ± 1.32 a
LSD*	2.51	2.21	2.01	1.97	1.11

Means followed by the same letter in each column are not statistically different (*P < 0.05)

LSD*= Least significant difference

S.E*= Standard error

ppm*= Parts per million

Effect of antibiotics on the diameter of inhibition zone

Xanthomonas oryzae pv. *oryzae* was efficiently controlled by all the antibiotics with a varying degree at different concentrations. The maximum diameter of inhibition zone (28.31 mm) was showed by the Chloramphenicol at 100 ppm concentration while all other concentrations viz., 75, 50 and 25 also gave best results with the inhibition zone (19.84, 13.96, 8.82 mm) respectively, with higher efficacy. The second best sensitive antibiotic to bacteria was ampicillin trihydrate which gave maximum inhibition zone (25.02 mm) at 100 ppm concentration, followed by the streptomycin sulphate with the average inhibition zone of (24.99 mm) at 100 ppm concentration. It was noticed that all the antibiotics performed well at their maximum doses and gave better inhibition of the pathogen. Benzylpenicillin and Kanamycin sulphate did not perform well at all the concentration and proved to be the least effective antibiotics against the *Xanthomonas oryzae* pv. *oryzae* with the least (17.80, 13.50 mm) inhibition zones (Table 2).

Table 2. Effect of various antibiotics against *Xanthomonas oryzae* pv. *oryzae* with the average inhibition zone on nutrient agar plates.

	Diameter of zone of inhibition (mm ± S.E*)				
Concentartion ppm*	Streptomycin Sulphate	Kanamycin sulphate	Chloramphenicol	Ampicilin trihydrate	Benzylpenicillin
25	7.93 ± 0.64 c	5.53 ± 0.48 c	11.82 ± 0.52 c	10.59 ± 0.56 c	5.84 ± 0.62 b
50	15.89 ± 0.69 b	9.64 ± 0.29 b	13.96 ± 1.14 c	12.78 ± 1.14 c	8.31 ± 0.30 b
75	18.60 ± 0.63 b	11.63 ± 0.35 a	19.84 ± 0.33 b	18.56 ± 0.81 b	10.27 ± 0.55 b
100	24.99 ± 0.90 a	13.50 ± 0.46 a	28.31 ± 0.72 a	25.02 ± 0.70 a	17.80 ± 0.29 a
Control	2.04 ± 0.26 e	2.01 ± 0.26 d	2.00 ± 0.00 d	2.00 ± 0.00 d	2.01 ± 0.26 c
LSD*	4.71	2.21	4.11	4.79	5.11

Means followed by the same letter in each column are not statistically different (*P < 0.05)

LSD*= Least significant difference

S.E*= Standard error

ppm*= Parts per million

Discussion

Control measures through fungicidal, bactericidal or nematicidal chemistries are always effective for the killing or suppressing of the pathogen by blocking the potential metabolic pathway of the pathogen and bacteria are among those which are the crucial agents to cause huge deaths in the kingdom animalia, so the applications of broad spectrum antibiotics could be useful for the control of microbial diseases and may augment the expansion of resistant strains for both harmful and useful bacterial entities which were putting back the antibiotic susceptible bacteria (Son *et al.* 1997; Li *et al*. 1999). The most important disease of rice i.e., bacterial leaf blight could be managed by the applications of various chemistries like copper–soap mixture, copper–mercury fungicides and bordeaux mixture. The fact had been established that the prevailing soil conditions must have a greatest effect for the bacterial leaf blight development yet the economical and effective management strategies through chemistries had still to be developed for this holistic disease.

The reason might be that because of the pathogen diverse population is prevailing which is exclusively unpredictable regarding its sensitivity to broad spectrum antibiotics commonly utilized for the control of this disease (Aktar and Sarwar 1986). Gnanamanickam *et al*., (1999) reported significant differences among the broad spectrum antibiotics used used to check the growth of pathogen hence, the existence and development of drug resistant pathotypes seriously posed big problems in formulating specific control agents. Some broad spectrum antibiotics could control the growth of bacteria when they were applied to the culture media on daily basis and according to the visual observation chloramphenicol and penicillin/ dihydrostreptomycin were found to be very effective (Fitt *et al.,* 1992). The present study showed that 100 ppm concentration was found to be the best among all tested concentrations for the control of *Xanthomonas oryzae* pv. *oryzae* growth on nutrient agar and the 25 ppm concentration was observed to be the least supportive among all treatments.

Our results coincides with the findings of Casandra and Bernal (2007) which found the higher levels of chloramphenicol, ampicillin and streptomycin sulphate significantly suppressed the number of bacterial colonies. Our results also supported the observations of Erasmus *et al.* (1997) which proved chloramphenicol and streptomycin to inhibit the growth of pathogen significantly at higher concentrations of 75 and 100 ppm respectively. Similarly our findings are in accordance with khan *et al*., (2009) which proved chloramphenicol as the best antibiotic at higher concentration for the control of bacterial leaf blight of rice. Pereyra *et al*., (2009); Nayak

et al., (2008); Nithya *et al*., (2007) proved that elevated concentrations of the antibiotics responded significantly for the control of *Xanthomonas oryzae* pv. *oryzae* after 48 hrs.

Likewise, Haq *et al*., (2006) reported chloramphenicol and streptomycin proved to be effective at 100 ppm concentration with the increase in the concentration of the toxicant and an increase in the inhibition zone was notices at higher concentration of the antibiotics. These findings might be helpful for the control of bacterial leaf blight of rice which had already become a big threat to the rice production in Pakistan. It is suggested that field experiments must be performed to prove the effectiveness of these broad spectrum antibiotics as there is a possibility of some variation in the field results because there are many factors which influence the chemical management of plant diseases in the field.

Conclusion

The use of antibiotics might play an important role for the management of holistic bacterial leaf blight disease of rice which is increasing day by day in Pakistan to avoid the severe epidemics of the disease which cannot be ruled out in future.

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