Assessment of Genetic and Pathogenic Diversity of *Xanthomonas oryzae* pv. *oryzae* on High Yielding Local Variety, Tella Hamsa, from Farmer Fields in Gagillapur and Kompally, Andhra Pradesh

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ABSTRACT: Rice is one of the most important food crops of the world which is grown in various agro climatic conditions and it encounters several biotic and abiotic stresses. Among biotic stresses bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* is the major destructive disease in the world. There is no chemical effective against this disease, so growing resistant varieties is the only way to decrease the losses caused by the disease. To develop durable resistance varieties in the particular area under biotic stress conditions necessitates evaluation of rice genotypes. The present study revealed significant fingerprinting variations observed among the 44 *Xanthomonas oryzae* pv. *oryzae* isolates from Tella Hamsa genotype collected from different areas, Gagillapur and Kompally. In addition, much diverse pathotypic variation or virulence pattern was detected from set of differentials containing near isogenic lines and traditional cultivar differentials. Virulence data obtained from these differentials revealed that all of them were compatible with the resistance genes. However, these pathotypes were incompatible with the genes, *xa-5, Xa-10, xa-13* and *Xa-21* suggesting the possibility of deploying them for enhancing the resistance. Similar observations were reported in the research area of rice crop improvement. So, this study suggests the deployment of genes in combinations of two and three expressed wide spectrum of longevity resistance to bacterial blight pathogen.

KEY WORDS: Rice, DNA fingerprinting, UPGMA, disease resistance and pathotyping.

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*) causes bacterial blight (BB), one of the most serious diseases of rice. Rice is a worldwide staple food as well as a model grain for cereal biology (Ronald and Leung, 2002; Shimamoto and Kyozuka, 2002; Bennetzen and Ma, 2003). In India, the reported percentage of seed carrying the pathogen varies from less than 1 to 100% in the glumes of the rice seed (Kauffman and Reddy, 1969) grains, (Srivastava, 1967). BB is characterized by a high degree of race-cultivar specificity. There are over 30 reported races of isolates from several countries (Adhikari et al., 1999; Mew, 1987; Noda et al., 1996; 2001). A set of races identified in the Philippines using five differential rice cultivars has been used widely for identifying and classifying resistance to BB in other cultivars (Mew, 1987; Ogawa et al., 1991; Lee et al., 2003). It has been noted, however, that screening for resistance to pathogen populations specific to particular geographical locations and tailoring regional breeding programme accordingly are important (Mew, 1987). To expedite the analysis of a large number of samples, a simple and efficient DNA fingerprinting method based on polymerase chain reaction (PCR) using primers corresponding to the repetitive element IS*1112* has been developed (George et al., 1995). Outwardly directed oligonucleotides complementary to each end of the IS*1112* element were used to fingerprint pathogen strains, resulting in useful discrimination of Philippine strains of *X. oryzae* pv. *oryzae* (George et al., 1995; 1997). Although Leach et al. (1992) detected a strong association between RFLP types and pathotype groupings, the relationship between lineages and pathotypes appears to be complex (Nelson et al., 1994).

MATERIALS AND METHODS

Plant materials

Seeds of near isogenic lines carrying bacterial blight resistance genes singly and in different combinations. IRBB 1 (*Xa-1*) Ogawa (1987), IRBB 3 (*Xa-3*) Ogawa and Khush (1989), IRBB 4 (*Xa-4*), IRBB 5 (*xa-5*) Mew (1987), IRBB 7 (*Xa-7*), IRBB 8 (*xa-8*), IRBB 10 (*Xa-10*), IRBB 11 (*Xa-11*), IRBB 13 (*xa-13*) and IRBB 21 (*Xa-21*) Ogawa (1993) were obtained from the Directorate of Rice Research Institute, Hyderabad. The seeds of six cultivar differentials namely, Cempo Selak (*Xa-3*), IR 20 (*Xa-4*), DV 85 (*xa-5, Xa-7*), IR 8 (*Xa-11*), Java 14 (*Xa-1, Xa-3, Xa-12* and *Xa-hg*), and BJ 1 (*xa-13*) and seed materials of a local susceptible variety Taichung native (TN1) were also collected from the Directorate of Rice Research, India, for this study.

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Cultivation of rice plants

Seedlings of near isogenic lines, cultivar differentials and susceptible variety TN1 were grown in the green house on the wet bed. Four vigorously growing young seedlings were transplanted into plastic pots (13 cm x 13 cm x 14 cm) under flooded conditions in the greenhouse. After six days, a fertilizer mixture of N, P, K was supplied to the plants at the rate of 150–80–80 Kg/ha, as a basal dose in the form of urea, super phosphate and muriate of potash were incorporated into the soil by hand mixing.

Media Used

For present investigation, Wakimoto's medium was prepared with 0.5 gm of calcium nitrate, 1.82 gm of disodium hydrogen phosphate, 20.0 gm of sucrose, 5.0 gm of peptone, 0.05 gm of ferrous sulphate (The pH of the medium was adjusted to 6.8-7.2 before sterilization) and 18.0 gm of agar were added to one liter of distilled water and it is used for routine work (Karaganilla et al., 1973). For maintenance of the stock cultures skimmed milk medium was prepared with 100.0 gm of skimmed milk powder and 5.0 gm of mono-sodium glutamate were dissolved in one lit of distilled water. The pH of the medium was adjusted to 6.5 before sterilization (Nelson et al., 1994). For DNA extraction of the isolates nutrient broth was prepared with 3.0 gm of beef extract and 5.0 gm of peptone and were dissolved in one liter of distilled water. The pH of the medium was adjusted to 6.8-7.2 before sterilization (Mahadevan and Sridhar, 1996).

Isolation of genomic DNA

Genomic DNA of *X. oryzae* pv. *oryzae* strains were prepared from 5-ml nutrient broth cultures grown overnight. The bacterial cells were collected and then lysed in 650 µl of extraction buffer (100 mM Tris HCL, pH 8; 100 mM EDTA; 250 mM NaCl; 1% sodium dodecyl sulfate [SDS], wt/vol; 1% polyvinylpyrrolidone [PVP]-40, wt/vol) at 65°C for 30 min. DNA was isolated using a modified method with 100 µL of potassium acetate (3M potassium, 5M acetate, pH 4.8) and precipitated with isopropanol (George et al., 1997). The DNA was dissolved in sterile distilled water and relative concentration of DNA present in the solution was visually quantified by electrophoresis with DNA standards after staining with ethidium bromide.

PCR fingerprinting

Amplification was performed as described by Leach et al. (1992) and George et al. (1995), in a 25-µL volume containing 50 pico moles each of the two opposing primers, JEL1 (5'CTCAGGTCAGGTCGCC3') and JEL2 (5'GCTCTACAATCGTCCGC3') complementary to each end of IS*1112*, 20 ng of genomic DNA, 185 µM each of four dNTPs, approximately 2.5 units of *Taq* polymerase in a standard incubation buffer (Boehringer Mannheim) supplemented with 10 % dimethylsulfoxide (vol/vol), and 7.5 µl of Tris-HCl (pH 9.5) were used. The reaction mixture was overlaid with one drop of mineral oil, initially denatured for 1 minute at 94°C, and then subjected to 30 cycles of PCR (10 s of denaturation at 94°C, 1 minute of annealing at 62°C, and 8 minutes of extension at 65°C), and a final extension for 8 minutes at 65°C using a Thermal Cycler (Model: 2700) manufactured by Applied biosystems, Singapore. Aliquots of 15 µL of the reaction mixture containing the PCR products were loaded in a gel containing 1.5% agarose in 0.5 X Tris-borate buffer. Amplified products were resolved by electrophoresis for 4 h at 100 V, stained with ethidium bromide, and then photographed by using a gel documentation system (Syngene, United Kingdom) equipped with Similarity matrix soft ware (Syngene, United Kingdom). The banding pattern of each isolate was recorded in binary form with a numerical letter 1 representing the presence and 0 for the absence of each band. Similarity coefficients were calculated by using the un-weighed pair-group method using arithmetic averages (UPGMA). Bootstrap analysis using the computer program Winboot (Yap and Nelson, 1996) was used to assess the robustness of the groupings produced by cluster analysis. The phenograms were reconstructed by repeated sampling with replacement, and the frequency with which a particular grouping formed was used as a measure of the strength of the grouping (Felsenstein, 1985; Hedges, 1992; Yap and Nelson, 1996).

Pathotyping with a set of near-isogenic lines and cultivar differentials

A set of differentials consisting of near isogenic lines (NILs) and cultivars carrying single known bacterial blight resistance gene in the background of susceptible cultivar IR24 were used. (Ulaganathan et al., 1994; Shanti et al., 2001). A susceptible check cultivar TN1 was always maintained. Plants were raised in earthen pots and there were four plants in each pot. Three pots were maintained as replications for each isolate. Twenty-one-day-old plants were clip-inoculated with the bacterial strain following clip-inoculation method. Fifteen days after inoculation, the lesion length formed due to inoculation was measured with a ruler. Reaction showing lesion length <5 cm were considered as resistant and >5 cm were considered as susceptible.

Method of inoculation

Inoculations were done by a clip method at the maximum tillering stage, about 30 days after transplanting

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(Kauffman et al., 1973). Pairs of scissors was dipped into the bacterial suspension and the tips of the leaves were clipped. Individual clones or cultures were inoculated on all the leaves of four plants which composed of approximately 25-30 leaves. The plants were cultivated in a net house under natural photoperiodic conditions. Infected leaves were collected after 7-10 days of inoculation for further study. For each inoculation, this step was replicated three times.

Disease severity assessment

The mean of all the lesion grades of each cultures or clone on the variety inoculated was calculated by 0-9 scale expressed as disease score with the help of a ruler. For race typing, the host was classified as resistant, if the mean lesion length was between 0 and 5 cm. Lesion lengths greater than 5 cm were classified as susceptible reaction.

RESULTS

Molecular analysis of Tella Hamsa pathogen population

To understand whether or not the pathogen occurring on a variety grown in a different fields exhibits any variation, a sub-population of 44 single mother colony isolates were obtained from a modern high yielding cultivar Tella Hamsa grown in and farmers field at Gagillapur and Kompally, Rangareddy, Secunderabad in the dry season of 2007. The analysis of fingerprints of these isolates was revealed separately by constructing separate dendrogram.

(i) Tella Hamsa pathogen population from Gagillapur village

The fingerprints of Tella Hamsa isolates from Gagillapur village revealed the presence of 21 haplotypes of the bacterial pathogen. Representative fingerprints of set of these isolates are shown in figuer 1 and dendrograms was constructed as figuer 2. A total of two clusters, major and minor, were detected at a similarity level of 12 %. Of these, the major cluster consisted of 19 isolates and minor one comprised of two individual/single isolates. However, at 25 % similarity level in dendrogram we got 7 haplotypes, out of that five are individual isolates (TH *Xoo*- 42, 43, 50 40, 47). Whereas other two groups of haplotypes consist of five isolates (TH *Xoo*- 44, 41, 48, 33 and 36), and 11 isolates, respectively. When we considered one representative isolate at 25 % similarity level, all the isolates formed into two clusters of which one contains 16 isolates and the other has five individual/single isolates.

(ii) Tella Hamsa pathogen population from Kompally village

The fingerprints of Tella Hamsa isolates from Kompally village reveal the presence of 23 haplotypes of the bacterial pathogen. Fingerprints of set of these isolates are shown in figuer 3 and a dendrogram was constructed with a set of fingerprints of Tella Hamsa isolates (Fig. 4). A total of 3 clusters, two major and one minor, were detected at a similarity level of 12 %. Of these, major clusters consisted of 11 isolates (TH *Xoo*- 57, 69, 59, 64, 66, 56, 58, 63, 61, 68, 72) and 5 isolates (TH *Xoo*- 52, 53, 54, 60, 71), respectively; minor cluster comprised of two isolates only. And the remaining five are individual/single haplotypes.

Virulence analysis for the Tella Hamsa population from Gagillapur village

The virulence pattern of seven representative isolates of Tella Hamsa population from Gagillapur and Kompally village were pathotyped on two sets of differentials consisting of near-isogenic lines carrying single known resistance genes and cultivar differentials consisting of different genotypes with different resistance genes.

(i) Near Isogenic Lines

These isolates were grouped into four pathotypes (XA-161, XA-223, XA-291, and XA-302) and were present in different proportions in the sub-population. Pathotype XA-161 consisted of a maximum of three isolates and it was compatible to all the single resistance genes (*Xa-1*, *Xa-2*, *Xa-4*, *Xa-7*, *xa-8* and *Xa-10*) except *Xa-3*, *xa-5, xa-13 and Xa-21*, while the pathotype XA-223 consisted of a maximum of 3 isolates incompatible to *Xa-3, xa-5, Xa-10, xa-13 and Xa-21* genes (Table 1).

(ii) National Cultivar Differential

The virulence pattern of the same seven representative isolates was passed to national cultivar differential set. These isolates were grouped into a total of three pathotypes, xa-2, xa-4, and xa-13 when pathotyped using the national cultivar differentials. Pathotype xa-4 consisted of a maximum of four isolates and it was compatible to *Xa-11*, *Xa-4*, *xa-13* and *Xa3* and incompatible to *xa-5*, *Xa-7* and *Xa-1*, *Xa-3*, *Xa-12*, *Xa-hg* (Table 2).

Virulence analysis for the Tella Hamsa population from Kompally village

(i) Near Isogenic Lines

A select set of eight isolates of this Tella Hamsa population was pathotyped on two sets of differentials consisting of near-isogenic lines carrying single known resistance genes and cultivar differentials consisting of

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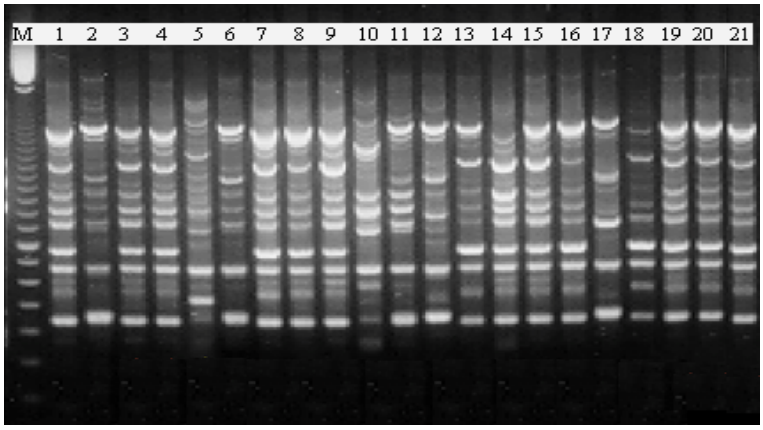


Fig. 1. Polymerase chain reaction fingerprint patterns of *Xanthomonas oryzae* pv. *oryzae* strains generated with IS *1112*-based primers (JEL1/JEL2). Lane M represents the 1 Kb ladder and followed by Lanes 1 to 21 containing the fingerprints of different Tella Hamsa isolates detected in the bacterial population from farmers field of Gagillapur village: TH *Xoo.* No. 31, TH *Xoo.* No. 32, TH *Xoo.* No. 33, TH *Xoo.* No. 34, TH *Xoo.* No. 35, TH *Xoo.* No. 36, TH *Xoo* .No. 37, TH *Xoo.* No. 38, TH *Xoo.* No. 39, TH *Xoo.* No. 40, TH *Xoo.* No. 41, TH *Xoo.* No. 42, TH *Xoo.* No. 43, TH *Xoo.* No. 44, TH *Xoo.* No. 45, TH *Xoo.* No. 46, TH *Xoo.* No. 47, TH *Xoo.* No. 48, TH *Xoo.* No. 49, TH *Xoo.* No. 50, and TH *Xoo.* No. 51.

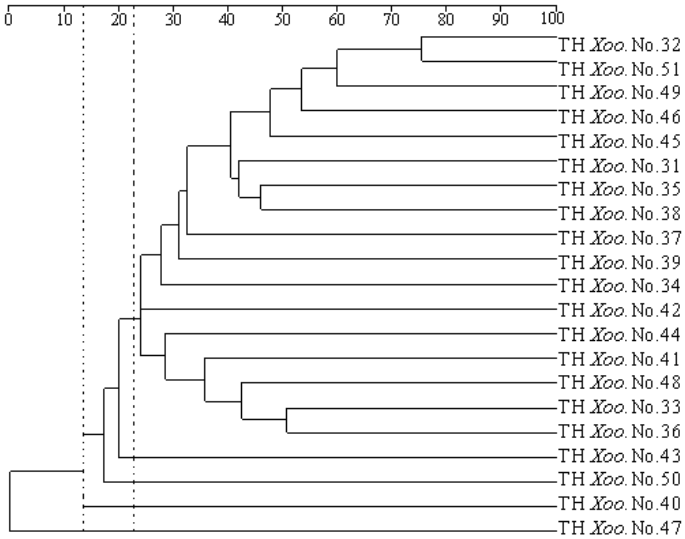


Fig. 2. Dendrogram constructed with UPGMA on the basis of IS*1112* repetitive element-based polymerase chain reaction fingerprint data for a collection of 21 Tella Hamsa isolates of *Xanthomonas oryzae* pv. *oryzae* from farmers field of Gagillapur village, Rangareddy. Homology level of 12 %, 22 % were marked with broken lines vertically.

Table 1. Pathotyping of a select set of bacterial blight isolates obtained from cultivar Tella Hamsa from Gagillapur farmers field using a set of near-isogenic line differentials (Data are lesion length in cm, lesion length >5 cm is considered as susceptible).

Pathotype	Isolate No.	*Xa-1*	*Xa-2*	*Xa-3*	*Xa-4*	*xa-5*	*Xa-7*	*Xa-8*	*Xa-10*	*xa-13*	*Xa-21*
XA-161	TH Xoo. No.36	S	S	R	S	R	S	S	S	R	R
XA-161	TH Xoo. No.39	S	S	R	S	R	S	S	S	R	R
XA-161	TH Xoo. No.40	S	S	R	S	R	S	S	S	R	R
XA-223	TH Xoo. No.42	S	S	R	S	R	S	S	R	R	R
XA-302	TH Xoo. No.43	R	S	R	S	R	S	S	R	R	R
XA-223	TH Xoo. No.47	S	S	R	S	R	S	S	R	R	R
XA-291	TH Xoo. No.50	S	S	R	R	R	S	S	R	R	R

Table 2. Pathotyping of a select set of bacterial blight isolates obtained from cultivar Tella Hamsa from Gagillapur farmers field using a set of traditional cultivar differentials (Data are lesion length in cm, lesion length >5 cm is considered as susceptible).

Pathotype	Isolate No.	*Xa-11*	*Xa-4*	*xa-13*	*xa-5, Xa-7*	*Xa3*	*Xa-1, Xa-3, Xa-12, Xa-hg*
xa-4	TH Xoo. No.36	S	S	S	R	S	S
xa-2	TH Xoo. No.39	S	S	S	S	S	R
xa-4	TH Xoo. No.40	S	S	S	R	S	S
xa-4	TH Xoo. No.31	S	S	S	R	S	S
xa-13	TH Xoo. No.43	S	S	S	R	S	R
xa-4	TH Xoo. No.47	S	S	S	R	S	S
xa-13	TH Xoo. No.50	S	S	S	R	S	R

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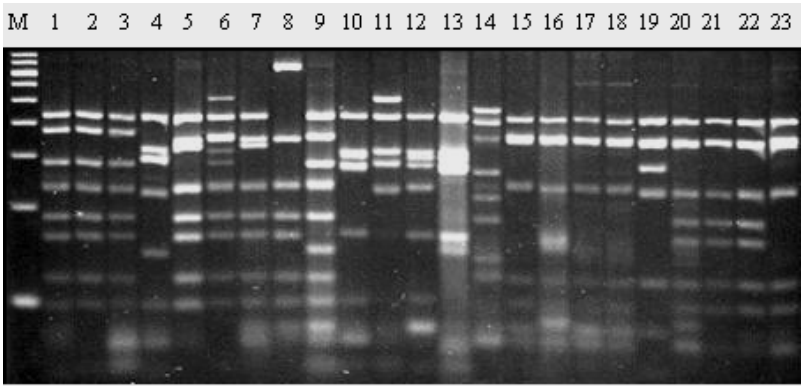


Fig. 3. Polymerase chain reaction fingerprint patterns of *Xanthomonas oryzae* pv. *oryzae* strains generated with IS *1112*-based primers (JEL1/JEL2). Lane M represents the 1 Kb ladder and followed by Lanes 1 to 23 containing the fingerprints of different Tella Hamsa isolates detected in the bacterial population from farmers field of Kompally village: TH *Xoo.* No. 52, TH *Xoo.* No. 53, TH *Xoo.* No. 54, TH *Xoo.* No. 55, TH *Xoo.* No. 56, TH *Xoo.* No. 57, TH *Xoo.* No. 58, TH *Xoo.* No. 59, TH *Xoo.* No. 60, TH *Xoo.* No. 61, TH *Xoo.* No. 62, TH *Xoo.* No. 63, TH *Xoo.* No. 64, TH *Xoo.* No. 65, TH *Xoo.* No. 66, TH *Xoo.* No. 67, TH *Xoo.* No. 68, TH *Xoo.* No. 69, TH *Xoo.* No. 70, TH *Xoo.* No. 71, TH *Xoo.* No. 72, TH *Xoo.* No. 73, and TH *Xoo.* No. 74.

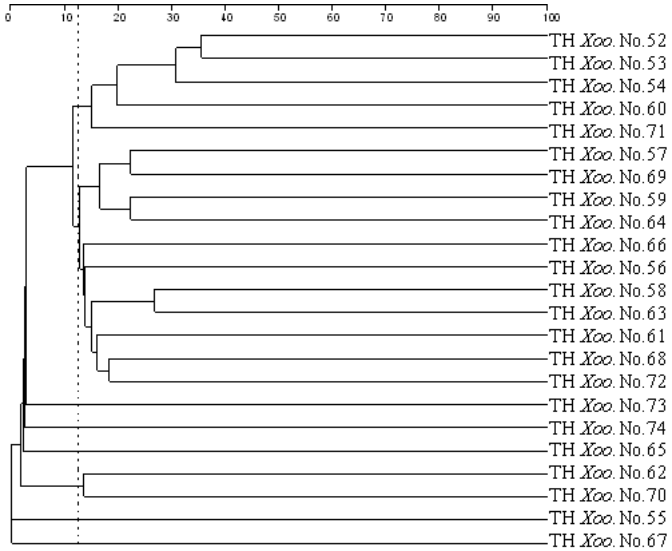


Fig. 4. Dendrogram constructed with UPGMA on the basis of IS*1112* repetitive element-based polymerase chain reaction fingerprint data for a collection of 23 Tella Hamsa isolates of *Xanthomonas oryzae* pv. *oryzae* from farmers field of Kompally village, Rangareddy. Homology level of 12 % was marked with broken lines vertically.

different genotypes with different resistance genes. These isolates were grouped into four pathotypes (XA-161, XA-223, XA-239, XA-280 and XA-291) and were present in different proportions in the sub-population. The pathotype XA-161 consisted of a maximum of 3 isolates and it was compatible to all the single resistance genes (*Xa-1, Xa-2, Xa-4, Xa-7, xa-8* and *Xa-10*) except *Xa-3, xa-5, xa-13 and Xa-21*, while the pathotype XA-239 was incompatible to *Xa-3, Xa-4, xa-5, xa-13 and Xa-21* genes (Table 3).

(ii) National Cultivar Differential

The virulence pattern of the same senen representative isolates was passed to national cultivar differential set. These isolates were grouped into a total of three pathotypes, xa-2, xa-4, and xa-13 when pathotyped using the national cultivar differentials. Pathotype xa-2 consisted of a maximum of four isolates and it was compatible to *Xa-11, Xa-4, xa-13, Xa3* and *xa-5, Xa-7* and incompatible to *Xa-1, Xa-3, Xa-12, Xa-hg* genes (Table 4).

CONCLUSIONS

Bacterial blight pathogen populations infecting Tella Hamsa variety from Gagillapur village were not different from Tella Hamsa variety growing in Kompally area. This is because influence of season and region may have a minor effect on the occurrence of the bacterial strains. The pathogen population from a very diverse set of rice genotypes grown in different areas exhibited a greater level of diversity suggesting that the host genotypes exert a major influence on the pathogen population. In addition to the pathotyping data, it shows the importance of the resistance genes, *xa-5, Xa-10, xa-13* and *Xa-21*, could be used for deployment to enhance rice resistance to the disease. So, this study suggests the presence of more than one gene in line contributed to a durable level of resistance to this disease.

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Table 3. Pathotyping of a select set of bacterial blight isolates obtained from cultivar Tella Hamsa from Kompally farmers field using a set of near-isogenic line differentials (Data are lesion length in cm, lesion length >5 cm is considered as susceptible).

Pathotype	Isolate No.	*Xa-1*	*Xa-2*	*Xa-3*	*Xa-4*	*xa-5*	*Xa-7*	*Xa-8*	*Xa-10*	*xa-13*	*Xa-21*
XA-291	TH Xoo. No.53	S	S	R	R	R	S	S	R	R	R
XA-239	TH Xoo. No.55	S	S	R	R	R	S	S	S	R	R
XA-239	TH Xoo. No.62	S	S	R	R	R	S	S	S	R	R
XA-280	TH Xoo. No.65	S	S	R	S	R	S	R	R	R	R
XA-161	TH Xoo. No.67	S	S	R	S	R	S	S	S	R	R
XA-161	TH Xoo. No.72	S	S	R	S	R	S	S	S	R	R
XA-223	TH Xoo. No.73	S	S	R	S	R	S	S	R	R	R
XA-161	TH Xoo. No.74	S	S	R	S	R	S	S	S	R	R

Table 4. Pathotyping of a select set of bacterial blight isolates obtained from cultivar Tella Hamsa from Kompally farmers field using a set of traditional cultivar differentials (Data are lesion length in cm, lesion length >5 Cm is considered as susceptible).

Pathotype	Isolate No.	Xa-11	Xa-4	xa-13	xa-5, Xa-7	Xa3	Xa-1, Xa-3, Xa-12, Xa-hg
xa-13	TH Xoo. No.53	S	S	S	R	S	R
xa-2	TH Xoo. No.55	S	S	S	S	S	R
xa-2	TH Xoo. No.62	S	S	S	S	S	R
xa-2	TH Xoo. No.65	S	S	S	S	S	R
xa-13	TH Xoo. No.67	S	S	S	R	S	R
xa-13	TH Xoo. No.72	S	S	S	R	S	R
xa-2	TH Xoo. No.73	S	S	S	S	S	R
xa-4	TH Xoo. No.74	S	S	S	R	S	S

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印度安得拉邦 Gagillapur 及 Kompally 農場中之稻米的地區性高產量品系"Tella Hamsa"病原菌，*Xanthomonas oryzae* pv. *oryzae*，的遺傳及致病性之多樣性的評估

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摘要：稻米是全世界最重要的糧食作物之一，並且栽植於不同的農業氣候區域，受到不同的生物性及非生物性的壓力。在生物性的壓力中，由 *Xanthomonas oryzae* pv. *oryzae* 所引起的細菌性葉枯病是最主要的毀滅性疾病，目前並無有效的化學藥劑可以對抗此病。因此提高稻米本身的抗病性是減少此病造成損害的唯一方法。若要於某些具高生物性壓力的地區，培養出具高耐受度的抗病品系，需要評估稻米的基因型。由來自 Gagillapur 及 Kompally 的 44 株地區性高產量品系"Tella Hamsa"，分離出 *Xanthomonas oryzae* pv. *oryzae* 的基因型，本研究顯示出 44 個分離株的 DNA 鑑識結果有明顯的變異性。除此之外，由相近的等基因型品系差別型及傳統品系差別型，其致病性或毒性具有高變異性。由這些差別型獲得的毒性資料顯示它們皆與這些抗病性基因相容。具抗病基因而與致病型不相容之稻米品系有 xa-5, Xa-10, xa-13 and Xa-21，顯示可藉由這幾個品系的基因型來誘發其他植株的抗病性。類似的報導亦在此稻米改良研究中出現過。因此本研究提出：可採用結合 2 至 3 種對細菌性枯葉病具長效抗病性的寬譜系來誘發稻米的抗病性。

關鍵詞：稻米、DNA 鑑識、UPGMA (不加權平均連結)、抗病性、致病型。

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