DIFFERENTIAL HYPERSENSITIVE RESPONSE OF NON-HOST SPECIES TO *XANTHOMONAS ORYZAE* PV. *ORYZAE* IN THENORTHWEST PAKISTAN

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خلاصہ

Hypersensitive response ایک دفاعی طریقہ کار ہے جو کہ مرض زاء حیاتیات کی نشو نما کو روکتا ہے۔ اس کی نشاندہی پودوں اور مرض زاء حیاتیات کے درمیان مطابقت پزیر تعامل اور گرد خلیات کی موت کی شکل میں ہوتی ہے۔ چاول ایشائی ممالک میں سب سے زیادہ استعمال ہونے والی غذا ہے لیکن پاکستان میں چاول کو مختلف بیماریاں لاحق ہیں۔ ان میں Hypersensitive responce کا معائینہ کرنے کیلئے ہم نے Xanthomonas oryzae pv oryzae کو تین مختلف میزبان پودوں کے ساتھ تجربہ کیا۔ یہ تجربہ Xanthomonas oryzae pv oryzae کے دس الگ نمونے اور تین میزبان پودوں پر مشتمل تھا۔ Hypersensitive response کی نشاندہی ایک مخصوص قسم کے بیکٹیریا کی موجودگی سے ثابت ہوا کہ Hypersensitive response کی اصل وجہ Xanthomonas oryzae pv oryzae کی موجودگی ہے۔ دتورا کے پودے میں سب سے زیادہ Hypersenitive response پایا گیا اور اس کی نشاندہی اس کے پتوں پر ایک مخصوص داغ سے کی گئی۔ دوسرے پودوں میں جیسے کہ Lamb−Quarter Milk−Thistle میں Hypersensitive response بہت کم پایا گیا۔

Abstarct

Hypersensitive response (HR) is a defensive mechanism system in the plant, which prevents the spread of pathogens. It characterized in form of cells death in the surrounding and produce a distinctive feature of resistance among various types of incompatible interactions between plant and pathogen. Rice is of one the most important food an Asian countries but Pakistan is suffering from the attack of many diseases particularly the bacterial leaf blight. To evaluate the hypersensitivity response of diverse isolates of *Xanthomonasoryzae*pv. oryzae (Xoo) from rice on different plant species. The experiment comprised of ten isolates of *Xanthomonasoryzae*pv. Oryzae along with three non-host species used for hypersensitivity response. To test the response of hypersensitivity, the bacterium was confirmed by Physiological and biochemical tests. All tests have shown the positive response for the bacterium and confirmed the causal organism as *Xanthomonasoryzae*pv. oryzae. Datura (*Daturawrightii)* had good hypersensitivity response in term of lesions size as compared to Lambs-Quarter (*Chenopodium album*) whereas Milk-Thistle (*Silybummarianum*) showed poor hypersensitivity response to isolates.

Introduction

In Pakistan, bacterial leaf blight (BLB) was reported for the first time and reported in all provinces of Pakistan (Mew and Majid, 1977; Aktar and Akram, 1987). It is one of the most devastating diseases which cause millions of tons of grain losses. World 90% of rice is cultivated only in an Asian country which is considered a staple food for approximately 2.7 billion people of the world (Salim *et al*., 2003). According to Khan *et al.*, (2000) the incidence of BLB is progressively increasing day by day in "Kaller" belt of Pakistan, which is well-known for rice farming. Recently, several races of BLB have been identified in Pakistan but this disease is more destructive in Asian countries during heavy rains of monsoon (Shazia *et al*., 2009). Rice is reduced up to 50% in the field due to the extreme severity of BLB and tillering stage the losses extend up to 10-20% (Mew and Majid, 1977) and disease causes a great threat in the economic sector.

Though, there are a number of diseases which affects the rice crop around the world. But due to their huge losses BLB *Xanthomonasoryzae* PV. oryzae, are considered one of the most dangerous disease comparable to other (Swings *et al*., 1990 and Mew, 1987). Rice crop is susceptible to this bacterium from seedling to mature stage. However, the disease is mostly expressed in two phases i.e. initially as 'kresek' and later on, the mature symptom appears as 'leaf blight' (Mizukami, 1956, 1961; Tabei and Mukoo, 1960). The initial symptoms of this disease consist of a little water soaked infection turn yellow, coalesce gradually and develop into an elongated lopsided lesion with a wavy margin. A small yellowish, bubble-shaped masses of bacterial ooze sometimes; may be seen on fresh leaves margin during moist condition. However, with the passage of time the lesions occupy an area of the leaf then the color turn into white and gradually secondary grayish growth appear (Tagami and Mizukami, 1962; OCTA, 1970; Ou, 1985). The plant produces panicle which is mostly sterile and contains immature grains. During severe infection, approximately 50% yield reduction has been documented (Mew *et al*., 1993) whereas, in mild infection, the losses have been noticed around 10-12% (Ou, 1985). Purposed this study was conducted to confirm the isolates of BLB from Swat District of Khyber Pakhtunkhwa (KP) and to record its hypersensitivity response on different non-host plant species.

Materials and Methods

Sampling

In order to isolate *Xanthomonasoryzae*pv. oryzae, the causal agent of BLB a survey was carried out at eleven (Table 1) different rice-growing areas at district Swat, Khyber Pakhtunkhwa (KP). The upper infected leaves showing a typical symptom of BLB were obtained at various location. The samples were labeled and preserved in ice-bucket to keep it fresh. These samples were brought to the Department of Plant Pathology, The University of Agriculture (UOP) Peshawar- Pakistan

Isolation of Pathogen

To isolate the causal agent of BLB, infected leaf samples (i.e. one gram) were cut into small pieces and were surface sterilized three times with 0.01% mercuric chloride. The samples surfaces were washed with sterilized distal water for 20-30 second. Leaf pieces were crushed in 0.85% sterile saline distal water and than one milliliter of suspension was moved to Petri plates containing Nutrient Agar (NA) media. These samples were accurately labeled and incubated for 72 hours at 30˚C. When the colonies with entire margins developed that were further sub-cultured by streaking method, to obtain a pure culture of *X. oryzae*pv. oryzae.

Identification of Bacterium

Potassium Hydroxide Test: Gram staining is the most validated assay of 3% KOH (Suslow *et al.,* 1982). Aseptically, colonies of bacteria were picked with the help of sterilized tooth-pick which were placed in a droplet of 3 % KOH solution and mixed for 15-20 seconds. In case of a gram-negative bacterium, it produces viscous and thread like structure when a pickup with a tooth-pick while in case of a gram-positive bacterium, it scattered on the slide without any thread (Rye, 1940).

Catalase Test: The bacteria were aseptically taken with the help of tooth-pick and place in a drop on a slide 3% of hydrogen peroxide (H2O2) solution and stirred for 5-10 seconds. In the case of Gram-negative, bubbles were produced in suspension whereas Gram-positive, produced no bubbles in the suspension (Schaad *et al.,* 2001).

Starch Hydrolyses Test: For starch hydrolyzes test we take twenty-eight grams of NA and dissolved in sterilized distal water while two gram of starch was dissolved in 10 ml of sterilized distal water separately. Both of these mixtures were added to NA media with constant stirring. Around 100 ml of Mixture was sterilized at 115˚C for 10 minutes. Media was aseptically poured into plates; each isolate was transferred to plate and these plates were incubated at 27˚C for seven days. The Lugol's iodine was flooded to each bacterial plate; which is prepared by dissolving one gram iodine along with two gram potassium iodide in 300 ml distilled water. The clear regions around the colonies showed the confirmation of starch hydrolyzes (Cowan, 1974).

Hypersensitive Response: The hypersensitivity response of different isolates was evaluated on non-host plant;Datura (*Daturawrightii*), Lamb Quarter (*Chenopodium album*) and Milk thistle (*Silybummarianum).* These plants were grown in various earthen pots containing loamy soil. Approximately 108-109cfu ml-1 of freshly cultured bacteria were injected onto the abaxial surface of indicator plant leaf with a hypodermic syringe at the 5-6-leaf stage. Inoculated plants were then kept in a moist chamber for a few hours to promote symptom development which was later shifted to the screen house. Controls were similarly inoculated with sterile distal water. The complete collapse of tissue after 24 hours, followed by necrosis was interpreted as a positive reaction (Klement and Goodman, 1967).

Results

Isolation of *Xanthomonasoryzae*pv.oryzae: All the plates inoculated with the samples were checked for the presence of *Xanthomonasoryzae*pv. *Oryzae* (Fig 1 and 2). The characteristic yellow colored, shiny, dome shaped colonies of the bacterium were observed in the plates (Fig. 3).

Biochemical Charactreization Tests

Potassium Hydroxide Test: The test was performed to demonstrate and the bacterium is a Gram negative i.e. the isolates produced thread like slime when pickup by toothpick and confirmed that the bacteria is *Xanthomansoryzae*pv. *Oryzae*(Table 2 and Fig. 3).

Catalase Test: The test was conducted to justify the 3% KOH test and confirm that bacteria is *Xanthomonasoryzae*pv. oryzae. The catalase test showed that a bacterium is Gram-negative i.e. isolates produced bubbles when it was stirred with the help of toothpick (Table 2 and Fig. 3).

Starch Hydrolyses Test: The NA plates were inoculated by isolates *Xanthomonasoryzae*pv. oryzae. After seven days the plates were stained with Lugol's iodine. All tested isolates, one isolate showed negative response whereas the remaining 10 isolates were a positive response (Table 2 and Fig. 3).

Hypersensitive Response

A set of 10 isolates obtained from the rice leaf extract were inoculated for a hypersensitive response on three different plant species. Lesion of collapsed and water soaked were observed for 24-hours, 48-hours and 72-hours followed by dry, light brown necrosis water soaked tissues within 24, 48 and 72 hours. Isolates with such response were ranked HR- positive. Out of the three indicator plants, two showed a positive result, while the third showed HR-negative (Fig. 1). These isolates were either a virulent strain for third indicator plant or may be host to the bacterium.

Table. 1Isolates representing different areas and cultivars of district swat

S. NO	Location	Isolates no.	Cultivar
1	Fizaghat	Xoo-1	JP5
2	ARI Mingora	Xoo-2	Swat-2
3	Malamjaba	Xoo-3	BAS-385
4	Shamozia	Xoo-4	JP5
5	KhawazaKhela	Xoo-5	FakhriMalakand
6	Matta	Xoo-6	JP5
7	Nawakaly	Xoo-7	Dilrosh
8	Charbagh	Xoo-8	Swat-1
9	Kabal	Xoo-9	BAS-385
10	Barikot	Xoo-10	JP5*BAS-385
11	Saidu Sharif	Xoo-11	FakhriMalakand

Table. 2Biochemical tests for the identification of *Xanthomonasoryzae*pv. *oryzae*, isolated from different leaf samples

S.	Isolates No.	KOH	Catalase test	Starch Hydrolyses
1	Xoo-1	-	+	+
2	Xoo-2	-	+	+
3	Xoo-3	-	+	+
4	Xoo-4	-	+	+
5	Xoo-5	-	+	+
6	Xoo-6	-	+	+
7	Xoo-7	-	+	+
8	Xoo-8	-	+	+
9	Xoo-9	-	+	+
10	Xoo-10	-	+	+
11	Xoo-11	+	-	-

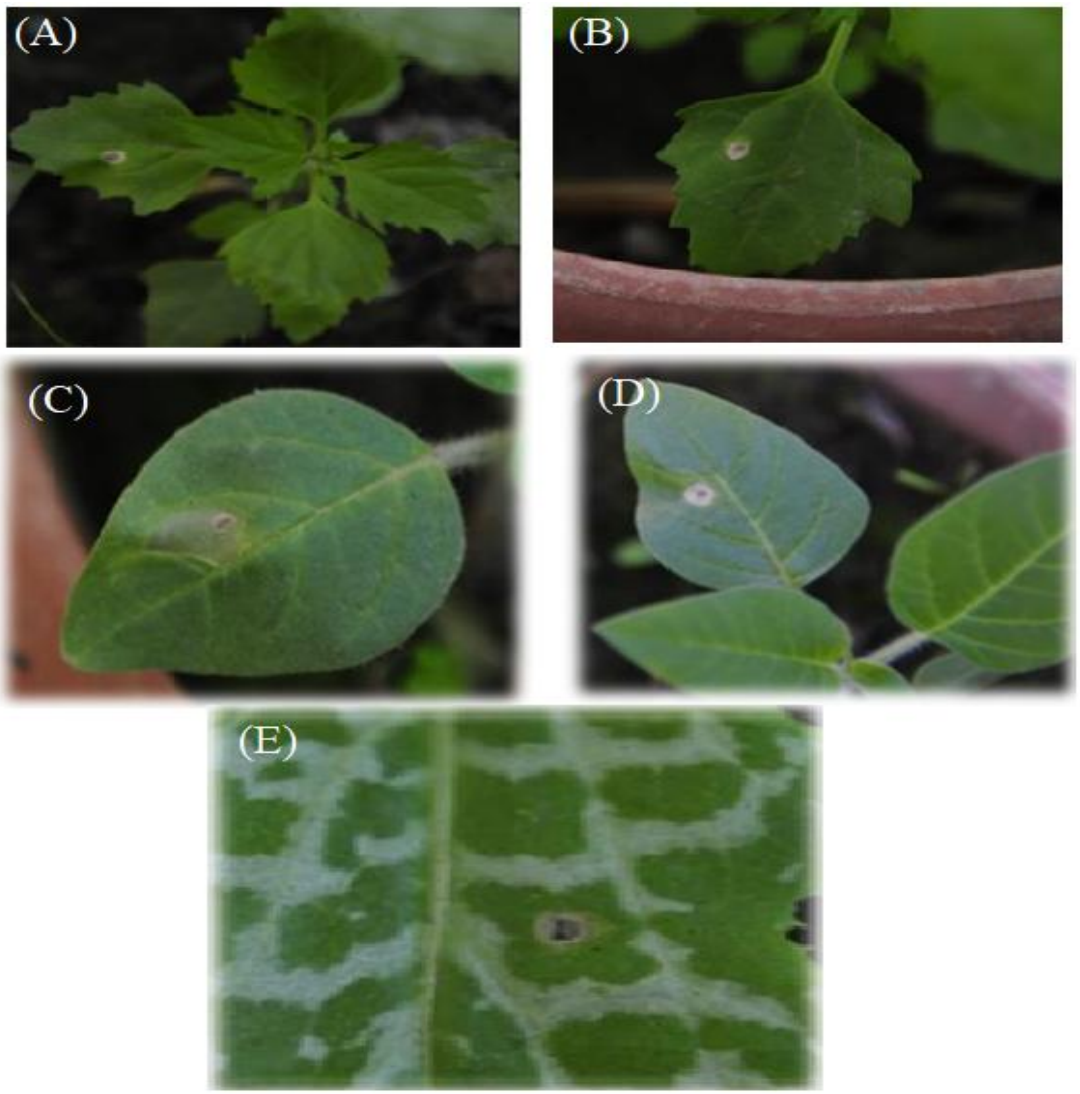

Fig.1. Showed different hypersensitive response of *Xanthomonasoryzae*pv. *oryzae* on (A and B) Lamb quarter (*Chenopodium album)* (C and D) Milk Thistle(*Silybummarianum)* and (E) datura (*Daturawrightii).*

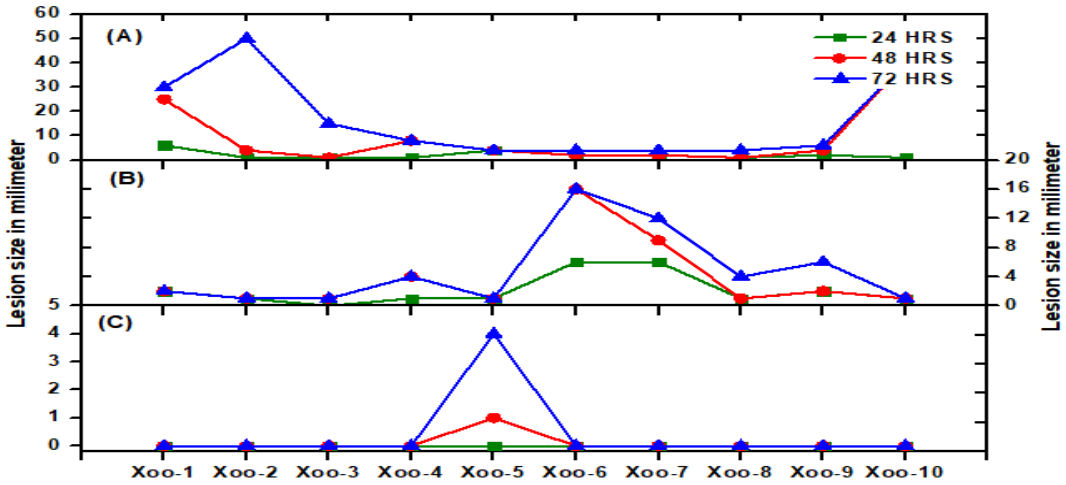

Fig.2. Hypersensitivity response and lesions size (mm) isolates on (A) Datura plants (*Daturawrightii*), (B) Lamb quarter (*Chenopodium album)* and (C) MilkThistle (*Silybummarianum)* from Swat valley of Pakistan.

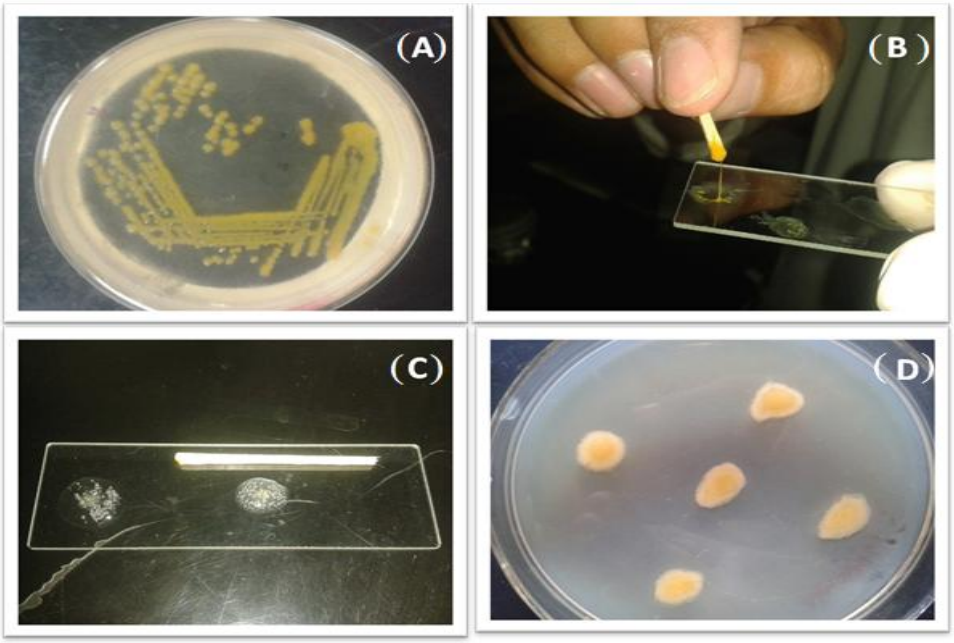

Fig.3. Physical and biochemical tests confirmed *X. oryzae*pv. *oryzae,* the causal agent of rice (A) yellow, round, doomed shape and shiny colonies of BLB (B) KOH test produce threat for BLB bacterium (C) Catalase test showed positive response and (D) Starch hydrolyses test produced clear zone.

Discussion

The present study was conducted to measure the hypersensitivity response of different isolates of BLB of rice on different indicator plants. Studies showed that *Xanthomonas* produced mucoid, domed and shiny yellow colonies on NA media (Cruz *et al*., 1984; Saddler 2012). Comparable to this study our analysis also showed similar characteristics. In addition, the biochemical analysis demonstrated that the bacterium observed in the present finding was gram negative. Further, the starch hydrolysis revealed positive results with the clear zone. Similar, results were also observed in the Swings *et al*., (1990). They reported that *Xanthomonas* produce mucoid, doomed, shiny yellow colonies on NA media (Cruz *et al*., 1984). Such colonies were consistently isolated on NA medium of our study. Different biochemical tests were conducted in order to characterize the bacterium on a biochemical basis. Based on KOH and catalase test the bacterium of our study showed characteristics of negative bacterium whereas the starch hydrolysis was positive and produced a clear zone. This result was similar to the study of Swings *et al*., (1990).

The isolates *Xanthomonasoryzae*pv. oryzae which were recovered from the leaf extract of BLB. Ten isolates were inoculated in three different indicator plants; showed typical necrotic symptoms were recorded for 24, 48 and 72 hours respectively. All isolates shown the positive response for two indicator plant;Datura (*Daturawrightii*) and Lamb Quarter (*Chenopodium album*), whereas Milk thistle (*Silybummarianum*) had a very poor response to the pathogen of BLB (Fig No. 4, 5, and 6). The leaf tissue infiltrated with 2-3 ml concentration of virulent strains developed yellow discoloration necrosis after 24-72 hours. These finding supported the previous researchers work (Ou, 1972; Fakir and Ahmed, 1974; Sharma *et al*., 1987; Bhutta and Ahmed, 1994 and Faruq *et al*., 2014). Sharma *et al*., (1987) also detected a number of fungal species from the seed of Oryza sativa. The most common fungal species were *Fusariummoniliforme*, *Curvularialunata*,*Aspergillus flavus*, and *Rhizopus stolonifera*. Bhutta and Ahmed (1994) explored that *Xanthomonasoryzae*pv. Oryzae caused 11% and 12% infection in a variety of IRRI-6 rice at Lahore and Hyderabad locations respectively. According to He *et al*., (2010) *Xanthomonasoryzae*pv. Oryzae is a causal agent of rice bacterial blight disease which produced virulence factors including iron-chelating siderophores, extracellular enzyme and type III-secretion dependent effectors which are collectively causative and essential factors for virulence. The virulent strains did not exhibition-necrosis and delayed reaction behavior. It is suggestedby the study of Klementand Goodman (1967) that the death of cell occurs in surrounding of infection which later extended to other parts of the plant, recognized as systemic acquired resistance (SAR).

Conclusion and recommendation

We concluded that *Xanthomonasoryzae*pv.oryzae is the indicator bacterium found in Oryza sativa. The hypersensitivity response produced different lesion on indicator plants including Datura (*Daturawrightii*), Lambs-Quarter (*Chenopodium album*) and Milk-Thistle (*Silybummarianum*). Among these species, datura (*Daturawrightii*) showed good results to HR followed by Lambs-Quarter (*Chenopodium album*) whereas Milk Thistle (*Silybummarianum*) response was poor. This study will be helpful to use Datura as an indicator plant in the future study other than tobacco. This plant is easily approachable in the environment.

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