SEROLOGICAL AND PATHOLOGICAL EVALUATIONS OF *XANTHOMONAS ORYZAE* pv. *ORYZAE*, THE CAUSAL AGENT OF BACTERIAL BLIGHT OF RICE

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ABSTRACT

Six new serological groups IIb, VI, V, VII, VIII and IV were delineated with new monoclonal antibodies (MAbs) Xoo-7, Xoo-8, Xoo-9, Xoo-10 and Xoo-11 made to Asian strains and tested against 299 strains of *Xanthomonas oryzae* pv. *oryzae* representing various rice growing areas of the world. Serologically distinct populations were recognized by new MAbs among Indian and Nepalese strains of *X. o. oryzae.* No relationship between serogroups and virulence or serogroup and race was obtained, except that previously reported serogroups III, IV and V contained only weakly virulent, atypical strains of *X. o. oryzae* from the United States. Ninety seven percent of the typical *X. o. oryzae* strains reacted either with MAb Xoo-7 or a previously reported MAb, Xco-2, but no strain reacted with both. These MAbs identified lipopolysaccharide antigens and gave bright fluorescence in indirect immunofluorescence microscopy (IF). An immunofluorescence colony staining technique (IFC) also was developed for detection of *X. o. oryzae* from rice seed extracts with fluorescein isothyocyanate (FITC) conjugated MAbs Xco-2 and Xoo-7 after enrichment in a semiselective medium E. Colonies of typical strains of *X. o. oryzae* on medium E formed 3 to 4 days earlier than on other semiselective media. IFC enabled detection of as few as 44 cfu in 100 μl extract from 100 rice seeds (1% infestation rate) even when plates were crowded with contaminants. Since both antibodies give bright immunofluorescence, a mixture of these MAbs can be used with IFC to detect 97% of the typical strains of *X. o. oryzae.* Enrichment on medium E followed by identification of *X. o. oryzae* by IFC with two pathovar specific MAbs enhanced sensitivity of detection, and the method is useful for epidemiological and seed transmission studies of this pathogen.

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CHAPTER 1

INTRODUCTION

Rice, a staple food of millions of people, is cultivated over a sizable area in diverse geographical locations throughout the world with more than 90% of the total area concentrated in Asia (David, 1991). Insect pests and plant diseases limit rice production and contribute significantly towards the lower yields in many developing countries. Among about twelve bacterial diseases associated with rice, bacterial leaf blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* is considered one the most destructive diseases in many rice growing areas of the world (Goto et al., 1988; Mew, 1991).

Bacterial blight is one of the oldest known diseases of rice and was first seen by farmers in the Fukuoka area of Japan in 1884 (Ou, 1985). By 1960, it was reported from almost all the areas of Japan (Tagami and Mizukami, 1962). In other Asian countries, BB was noticed, in general, only after the high yielding, susceptible rice cultivars like TN1 and IR-8 were introduced (Mew, 1989), and at present it is known to occur in almost all the rice growing areas of the world including Asia (Ou, 1985), Latin America (Lozano, 1977), Sahelian countries of Africa: Mali, Niger and Upper Volta (Awoderu and John, 1984), Australia (Aldrick et al., 1973; Ramsey and Moffet, 1989), United States of America (Jones et al., 1989), and USSR (Mew, 1989).

The epidemic potential of BB is thoroughly documented and it can be seen in almost all the environments that suit rice cultivation. Considerable amount of information on various aspects of the disease is available and has been reviewed by many groups (Mew, 1987; Mew, 1989; Mew et al., 1993; Mizukami and Wakimoto, 1969; Ou, 1985; Tagami and Mizukami, 1962). Losses due to the disease depend upon the geographical location, climatic conditions and rice cultivar (Mew, 1987). In the irrigated and rainfed lowland areas of Asia that contribute to the 90% of world rice production (David, 1991),

BB is considered a major disease and is especially destructive during the heavy rains of the monsoon. It can be very destructive in other tropical areas where crops remain in fields through out the year. In double-cropped subtropical regions, BB occurs in both rice crops and in temperate environments, where rice is monocropped under irrigated conditions, it is commonly found during the rainy season from July to October (Mew, 1987; Mew, 1989; Mew et al., 1993). Losses up to 26% from Nepal (Adhikari and Mew, 1994) and between 6 to 60% from some states of India (Srivastava, 1967) have been reported. A total crop failure during the major BB epidemics was reported in some parts of India during 1979-80 (Durgapal, 1985). Rice yields also vary with the severity of the infection because 10 to 20% yield reductions were noticed with moderate infection and 50% reduction was recorded in severely infected fields (Mew, 1989). In extreme cases, plants do not produce grains. More often, however, disease occurs at the maximum tillering stage, and 10 to 20% yield losses are caused because of the reduced photosynthetic area and subsequent suppression in grain weight (Ou, 1985).

Bacterial blight is a vascular disease that is characterized by three types of symptoms: pale yellow leaves, kresek, and leaf blight (Mew, 1987). There appears to be some disagreement regarding the cause of the pale yellow leaf symptom that is usually observed on mature rice plants. Ou (1985) considered that the gradual build up of bacteria at the lower portion of the stem blocks the supply of nutrients and results in yellowing of leaves. Mew (1987), however, believed this to be a secondary effect of kresek or leaf blight and speculated involvement of a bacterial toxin. Although, reports on the isolation of toxins (Sreeramalu et al., 1983; Noda, et al., 1980), existence of extracellular polysaccharides (EPS) of *X. o. oryzae* in rice leaves (Miyazaki et al., 1975; Angadi, 1978) and the involvement of EPS in disease development through blockage of xylem vessels have been made (Tseng et al., 1983; Vidhyasekaran et al., 1989); the actual mechanism through which yellow leaf is produced, however, still remains to be established.

The kresek symptom, characterized as "the complete death of entire plant" was first reported in Indonesia (Reitsma and Schure, 1950). It is more commonly observed at the seedling stage. Transplantation injuries on roots and leaf tips are main portals for the bacterial invasion and kresek may be observed 1 or 2 weeks after transplanting when infected leaves become grayish-green and begin to fold up and roll along the midrib. Seedling death occurs within few weeks after infection (Ou, 1985). The bacterial strains that cause kresek are considered more aggressive than those that cause leaf blight (Devadath and Premalatha, 1970; Mew, 1987). Moreover, strains associated with kresek always induce wilt whereas the strains that cause leaf blight do not always produce kresek (Choi et al., 1978).

The name "leaf blight" is given the to disease because of the leaf blight symptom that is most commonly observed. The symptom is manifested mostly at the tillering stage, although, it may appear earlier (Mew, 1987). In temperate regions, it usually becomes noticeable in field at the heading stage. Hydathodes and wounds serve as ingress points for bacteria which multiply in the epithem for invasion of xylem vessels (Tabei, 1977; Horino, 1984; Mew et al., 1984). Hydathodes also were found to be involved in the specificity of interaction between rice and *X. o. oryzae* (Horino and Kaku, 1989; Huang and De Cleene, 1989). Lesions start as water-soaked stripes at the margins of the leaves enlarging both in length and width. As the disease advances, the entire leaf blade is covered with tannish gray to white lesions resulting in leaves with wavy margins. On susceptible cultivars, infected blades wilt and roll as the disease portion enlarges while the leaves are still green, extending to the sheath, and killing the entire blade in severe cases. The glumes on severely diseased plants show discolored spots surrounded by a water-soaked margin which turns grayish or yellowish white at maturity (Ou, 1985).

Rice seed, hulls, straw and stubble, volunteer rice plants, weeds such as *Leersia* spp., irrigation and field water, all have been implicated in the survival, spread and

transmission of *X. o. oryzae.* Reports of its survival, ranging from few days to several months and even up to 8 years, depending on the part of the plant infected or storage conditions, have been made (Awoderu and John, 1984; Dikin et al., 1993; Gonzalez et al., 1991; Lozano, 1977; Mew et al., 1989; Mizukami and Wakimoto, 1969; Ou, 1985; Rao and Kauffman, 1970; Reddy and Shang-zhi, 1989). The extent to which each of these sources contribute in the disease development, however, remains to be established. The implication of rice seed in the transmission of *X. o. oryzae* in some countries where disease was not reported before (Awoderu and John, 1984; Lozano, 1977) and the quarantine problems that are associated with the frequent international rice seed exchange of genetically improved cultivars (Leach and White, 1991) have made rice seed the target of transmission studies (Goto et al., 1988; Mew et al., 1989; Ou, 1985; Reddy and Shang-zhi, 1989). The conclusive evidence for transmission of the pathogen through seed and to the subsequent crop, however, remains to be clearly demonstrated (Mew et al., 1993)

The establishment of the bacterial nature of BB through pathogenicity tests, by Japanese scientists, about a century ago, not only eliminated the earlier speculations of this disease being physiological in origin and due to acid soil but also laid a foundation for intensive research on BB (Ou, 1985). The causal bacterium has undergone many changes in its nomenclature since then. According to Ou (1985), it was first described as *Bacillus oryzae* Hori & Bokura in 1911 and was later named *Pseudomonas oryzae* Uyeda & Ishiyama (Ishiyama, 1922). The names *Bacterium oryzae* (Uyeda & Ishiyama) Nakata and *Xanthomonas oryzae* (Uyeda & Ishiyama) Dowson were subsequently adopted afterwards in that order (Ou, 1985). With the revision in the International Code of Nomenclature of Bacteria, this organism became one of the pathovars of *X. campestris,* and the name *Xanthomonas campestris* pv. *oryzae* (Ishiyama 1922) Dye 1978, was established (Dye, 1978; Dye et al., 1980). Based on restriction length fragment polymorphism (RFLP) analysis with selected probes (Leach et al., 1990), fatty acid profiles (Stead, 1989),

monoclonal antibody reactions (Benedict et al., 1989), and the unique features found with both modern and classical techniques of bacterial characterization (Mew et al., 1993), once again, the BB pathogen was recently proposed as a separate species and the new name *Xanthomonas oryzae* pv. *oryzae* was proposed (Swings et al., 1990).

X. o. oryzae is a Gram negative rod with a single polar flagellum (Ishiyama, 1922; Ou, 1985) and grows aerobically at optimum temperatures ranging between 26 to 30 C. A wide pH range of 4.0 to 8.8 have been reported for the growth (Ou, 1985); however, the optimum pH is between 6 to 7 (Fang et al., 1957; Ou, 1985). The bacterium grows slowly on synthetic media and takes 5 to 7 days to produce even 1 to 2 mm diameter colonies on nutrient agar. Colonies are whitish yellow to straw yellow, convex, circular with entire and smooth margins; and the yellow pigments produced by the bacterium are insoluble in water (Ou, 1985). Peptone sucrose agar-PSA (Tsuchiya et al., 1982) is more commonly used for growing pure cultures of *X. o. oryzae.* In pure culture, it grows relatively fast on sucrose-containing media although it can also utilize other carbon sources (except fructose) to some extent. It also grows on media containing glutamic acid and cystine as nitrogen sources (Fang et al., 1957; Watanabe, 1963; 1966). Results regarding acid production from maltose, lactose, and dextrin; gelatin liquefaction, lipase activity, litmus milk reaction, NH_3 and H_2S production, starch hydrolysis, and pectolytic reaction are conflicting (Chakravarti and Rangarajan, 1967; Muko and Isaka, 1964; Sekhawat and Srivastava, 1968). The later studies clarified some of these differences and also pointed out that, despite phenotypic variation in various physiological and biochemical characters, strains of *X. o. oryzae* did not form distinct biochemical groups (Vera Cruz and Mew, 1989; Reddy and Ou, 1976; Hifni et al., 1975; Tsuchiya et al., 1982)

It has been clearly demonstrated that *X. o. oryzae* is highly variable with respect to its pathogenicity on differential rice cultivars (Mew, 1987). The variable nature of the pathogen was realized only after a previously resistant rice cultivar, Asakaze, succumbed

to BB in Japan in 1957. Thus, attempts were made to classify strains for virulence or to group rice cultivars on the basis of "resistance" when strains were inoculated on resistant, intermediate and susceptible cultivars (Ezuka and Horino, 1974; Kozaka, 1969; Kuhara et al., 1965; Kusaba et al., 1966; Mizukami, 1966; Tagami and Mizukami, 1962; Ou et al., 1971; Watanabe, 1966; Washio et al., 1966). Differences in virulence on rice cultivars also were reported from other countries (Buddenhagen and Reddy, 1972; Rao et al., 1971; Devadath & Padmanabhan, 1969). Pathogenic groups were recognized through distinct host-pathogen interactions on rice cultivars. Such groups were later termed "physiological races", which are defined as a group of strains that evokes a particular combination of susceptible (compatible) and resistant (incompatible) reactions when tested on a standard set of differential host cultivars (Mew et al., 1993). According to Mew (1987), many scientists doubt the existence of races in *X. o. oryzae* because interaction of *X. o. oryzae* with a rice cultivar does not always follow the "presence-versus-absence of disease phenomenon" (Mew and Vera Cruz, 1979) as observed with the races of some fungal pathogens like rice blast (Seshu et al., 1986). Strains of *X. o. oryzae,* were thus initially classified as "virulence" or "bacterial" groups. Even though such a phenomenon was not observed in rice cultivar-isolate interactions, the compatible and incompatible interactions were distinctively separable on the basis of "lesion lengths and lesion type" and, therefore, provided means to classify virulence or bacterial groups as "races" (Sato et al., 1977; Ezuka and Sakaguchi, 1978; Mew et al., 1982a). Using these criteria of classifying variation in strains of *X. o. oryzae*, races and groups have been described from the rice growing countries such as the Philippines, Japan, India, Indonesia, Thailand, Korea and Nepal and other rice growing areas of the world (Adhikari et al., 1994; Eamchit and Mew, 1982; Ezuka and Horino, 1974; Horino et al., 1981; Kozaka, 1969; Mew, 1987; Mew et al., 1982a; Mew et al., 1982b; Mew et al., 1993; Ogawa, 1983; Ogawa and Khush, 1989; Rehman et al., 1993).

The compatibility and incompatibility of interactions also was apparent in growth kinetics experiments of *X. o. oryzae* in rice leaves in which spread of bacteria in compatible interactions was more rapid and extensive than in incompatible interactions, and 100 to 1000 fold reductions in the final numbers of bacteria were observed in incompatible interactions (Barton-Willis et al., 1989). Antibacterial compounds also have been isolated from healthy leaves of susceptible and resistant cultivars (Horino and Kaku, 1989) and from leaves after exposure to avirulent strains of *X. o. oryzae* (Nakanishi and Watanabe, 1977). Some of these compounds were ligninlike polymers (Horino and Kaku, 1989) that accumulated rapidly in incompatible interactions and not in compatible interactions (Reimers and Leach, 1991). As observed by transmission electron microscopy, the bacteria in the compatible interactions were normal and appeared to have been actively reproducing, whereas in the incompatible interactions they were deformed due to the envelopment by fibrillar material of host origin. Bacterial multiplication was not affected by fibrillar material in compatible interactions because they were surrounded by electron-transparent zones, considered to be extracellular polysaccharides produced by virulent strains (Horino and Kaku, 1989; Huang and De Cleene, 1989). Based on the information gained in these studies, *X. o. oryzae* and rice-cultivar interactions were considered to follow the gene-for-gene concept (Flor, 1955; Ellingboe, 1976). Evidence supporting this hypothesis, at the molecular level was presented by Kelemu and Leach (1990), who identified and cloned an avirulence gene from a race 2 Philippine *X. o. oryzae* strain (PXO86) that is incompatible with rice cultivar Cas 209.

Despite the wealth of information that is available on this disease, BB still remains a great challenge to the scientists and a major threat to rice industry in many rice growing areas of the world, particularly Asia (Mew, 1989). Chemicals and antibiotics such as streptomycin, penicillin, cellocidin, aureomycin, chloromphenicol and blasticidin (Devadath, 1985; Hoa et al., 1984; Mary and Mathew, 1983, Ou, 1985) used to control

this disease are expensive and have not proved to be very effective. Integrated control using sanitation and other cultural practices (Devadath, 1989; Padmanabhan, 1983; Srivastava, 1967) combined with use of resistant rice cultivars and chemicals have therefore been recommended, since there is no single control measure available for this disease (Ou, 1985).

The use of resistant rice cultivars (Horino et al., 1981; Mew et al., 1982; Ogawa and Khush, 1989) has proved to be cost-effective for BB control (Mew et al., 1993) and has lead to the characterization of 21 resistance genes (Ogawa et al., 1991). However, resistance breaks down due to a continuous emergence of new races or shifts in the virulence of *X. o. oryzae* strains (Mew, 1987). Moreover, methods used to describe such races also vary from area to area. Therefore, a cultivar resistant to BB in one geographical location may not be resistant in other areas or even in the same area. For example, the pathotypes (races) described in Japan and the Philippines were found quite different because, IR-8, a cultivar susceptible to tropical strains, gave a resistant reaction to some of the Japanese strains (Horino et al., 1980 and 1981). Moreover, rice cultivars, resistant to Philippine and Japanese strains, were susceptible to Indian strains (Devadath & Padmanabhan, 1969). The average virulence of *X. o. oryzae* strains from Australia and Japan was lower than the strains from India, Sri Lanka, and Indonesia; and Asian strains, especially from India, were considered more virulent than the strains from other countries (Buddenhagen and Reddy, 1972). Thus, there is a need, not only for adequate understanding of the inherent variability of this organism but also for a continuous search and strategic deployment of resistance genes for better management of BB in the rice growing areas of the world. For that purpose, standardization of techniques, especially a set of standard differential rice cultivars that could identify and detect variation among the strains from all rice growing areas of the world, is highly desirable. While use of

differential rice cultivars for defining races of *X. o. oryzae* is useful, the method is too laborious and time-consuming.

Research efforts have been focused on relating the differences in virulence of *X. o. oryzae* to some biochemical, physiological, molecular and serological traits or to phage sensitivity. A study, using techniques of numerical taxonomy, on 103 phenotypic characters of 52 strains, revealed that the strains of *X. o. oryzae* were homogeneous regardless of their specificity to rice cultivars or geographical origin, and despite some phenotypic variation observed in various physiological and biochemical characters, strains of *X. o. oryzae* did not form distinct biochemical groups (Vera Cruz et al., 1989). Similar views were shared by some other groups also (Reddy and Ou, 1976; Hifni et al., 1975; Tsuchiya et al., 1982). While, in general, phenotypic characters did not seem to relate to the virulence (Goto, 1972; Tsuno and Wakimoto, 1983, Mew, 1987), some relationship between virulence and phage sensitivity was found using a suitable phage strain (Choi et al., 1981). Vera Cruz and Mew (1989) reported 8 lysotypes among 225 strains, representing 6 Philippine races, and indicated that the isolates of some race groups had similar phage sensitivity, whereas, other race groups overlapped. In general, however, it is believed that sensitivity to phages has no relationship to the virulence of *X. o. oryzae* strains (Goto, 1965; Ou, 1985).

Choi et al. (1980a) found 3 serovars with 160 strains of *X. o. oryzae* collected from different rice-growing areas in Asia with a serum that was not tested for its cross reactivity to other bacteria, and Wang et al. (1981) reported two serovars among 202 Chinese strains of *X. o. oryzae* in an indirect hemagglutination test. Lin et al. (1969) reported that they could easily differentiate between the virulent and weakly virulent strains of *X. o. oryzae* using a gel diffusion test with polyclonal antisera. Likewise, Choi et al. (1980b) found that virulent strains and weakly virulent strains formed separate serovars and also reported a pathovar-specific antigen using a polyclonal antiserum in a gel

diffusion test. In contrast, Addy and Dhal (1977), using gel diffusion, were unable to find any serological differences among 45 strains and reported only one serovar. Successive culturing of the organism on synthetic media resulted in loss of virulence due to mutation to avirulent types (Fujii, 1976; Reddy and Kauffman, 1977); colony type variants that were selected by growing a few strains of the bacterium on the medium over a six month period were also reported to be serologically different (Choi et al., 1981). No bacteriological, physiological tests, however, were performed by them to confirm the identity of the serologically different variants. Although reports regarding the serological variability in *X. o. oryzae* were made in these studies, the methods of analysis were either relatively insensitive, or specificity was not thoroughly studied.

Restriction fragment length polymorphism (RFLP) analysis was used to study polymorphism among 98 strains representing six races of *X. o. oryzae* from the Philippines and 27 RFLP types were found among these strains (Leach et al., 1990). Although the RFLP types within one race did not overlap with the RFLP types from other race(s), none of the RFLP types were diagnostic of the race despite the apparent genetic variability (Leach et al., 1992). It is obvious from studies conducted to date that the techniques that may replace the time-consuming method of race determination of *X. o. oryzae* by using differential rice cultivars are not likely to become available in the near future.

Detection and identification of a pathogen plays a key role in studying epidemiology, etiology, and spatial patterns of disease and also in management of disease (Leach and White, 1991). Detection of *X. o. oryzae* is difficult because of its slow-growing nature and poor ability to compete with the fast-growing contaminants that are often associated with the rice plant material (Mew et al., 1993). Efforts have been made to improve efficiency of isolation and detection of *X. o. oryzae* using several synthetic (Ou, 1985) and some semi-selective growth media (Yuan, 1990; Gonzalez et al., 1991; Di et al., 1991; Gnanamanickam et al., 1994), however, none of these media are useful for

detection of the typical, slow-growing *X. o. oryzae* strains. Although molecular biological techniques also are being tested or explored for this purpose, DNA probes hybridizing only with *X. o. oryzae* have not been found yet (Mew et al., 1993). A 2.4 kb repetitive DNA sequence cloned from *X. o. oryzae* was used to probe digested DNA, and the resulting RFLP patterns proved to be diagnostic of a pathovar *X. o. oryzae* (Leach et al., 1990). The use of this DNA probe for RFLP analysis to differentiate *X. o. oryzae* from *X. o. oryzicola* and pathovars of *X. campestris* has been proposed (Mew et al., 1993).

Serological tools have been used quite extensively for diagnosis in medical and agricultural sciences because they are simple and also require little equipment (Leach and White, 1991). Using polyclonal antibodies, in a direct immunofluorescence assay, Goto (1970) detected 1 X 10^5 cfu/ml of *X. o. oryzae* from rice-field water at the International Rice Research Institute (IRRI) experimental farm; however, specificity of the technique was not evaluated. Wang et al. (1980) detected *X. o. oryzae* from leaves and stubble with a polyclonal antiserum. Lee et al. (1982) also used polyclonal antisera raised to live *X. o. oryzae* cells in an indirect fluorescent antibody stain (IFAS) and identified 88 *X. o. oryzae* cultures from rice seeds and leaves gathered in 11 provinces and regions in China. The antiserum, however, cross-reacted with other pathovars of *X. campestris.* Although reports on the detection of *X. o. oryzae* were made, the methods of analysis were either relatively insensitive, or specificity was not thoroughly studied.

The hybridoma technology introduced by Kohler and Milstein (1975) has provided a revolutionary advance in the method of antibody production that eliminates many of the problems associated with polyclonal antisera. It has enabled us to produce unlimited quantities of monospecific-monoclonal antibodies that are ideal serological tools for taxonomic, diagnostic, epidemiological studies (Alvarez et al., 1985; Benedict et al., 1989; Benedict et al., 1990; Yuen et al., 1987), and structural and biochemical analysis of the plant-pathogenic organisms (Halk and De Boer, 1985). Using this technology, genus-

specific, pathovar-specific (Alvarez, et al., 1985; Benedict et al., 1989; Benedict et al., 1990; Alvarez et al., 1991) and even race and strain-specific antibodies (Wingate et al.,1990) have been generated. MAbs specific for *X. o. oryzae* proved to be useful in the detection of *X. o. oryzae* from artificially infected rice seeds (Benedict et al., 1989), for the identification of low virulence strains of *X. o. oryzae* from rice in United States (Jones et al., 1989), and to map and monitor the distribution of two strains representing two races of Philippine *X. o. oryzae* under field conditions (Roberts, 1991).

Based on the information on serological variability detected with MAbs, it was hypothesized that *X. o. oryzae* strains recovered from various rice growing areas of the world are serologically distinct. The present studies were initiated to determine serological variability among new strains of *X. o. oryzae* from India and Nepal not studied previously (Benedict et al., 1989). The second objective of the study was to determine the relationship between the serogroups and race or serogroups and virulence by testing representative strains from each serogroup on rice cultivars. The third objective was to characterize antigens recognized by the MAbs developed during the course of the present study and those reported previously (Xco-1, Xco-2 and Xco-5) (Benedict et al., 1989) using immunochemical and biochemical methods of analysis. The fourth objective was to utilize these MAbs for efficient detection and identification of *X. o. oryzae* from seed or other parts of the rice plant.

CHAPTER 2

New Serogroups of *Xanthomonas oryzae* pv. *oryzae* Delineated with Monoclonal Antibodies, and Analysis of the Antigens Recognized by These Antibodies.

ABSTRACT

Six new serological groups of *Xanthomonas oryzae* pv. *oryzae* were delineated with new monoclonal antibodies (MAbs) Xoo-7, Xoo-8, Xoo-9, Xoo-10 and Xoo-11 made to Asian strains and tested against 299 *X. o. oryzae* strains representing various rice growing areas of the world. None of the MAbs reacted with 106 other xanthomonads or 61 strains of other genera or species except MAbs Xoo-8 and Xoo-11 which reacted with a few strains of other pathovars of *X. campestris*. A subset of Indian and Nepalese strains of *X. o. oryzae* were distinguished from other Asian strains by positive reactions with MAbs Xoo-7 and Xoo-8. Ninety four percent of the strains reacting with these two MAbs were from Nepal and India. Among the 299 *X. o. oryzae* tested, 92% of the strains reacted either with MAb Xoo-7 or a previously reported MAb, Xco-2, but no strain reacted with both MAbs. Serogroups III, IV and V contained only weakly virulent, atypical strains of *X. o. oryzae* from the U.S.A. and India, but no other relationship between serogroups and virulence or serogroup and race was obtained. The antigens reconized by MAbs Xco-2, Xoo-7, Xoo-8 were identified as lipopolysaccharides whereas antigens recognized by a previously reported MAb Xco-1 was a protein moiety. MAbs Xoo-10 and Xoo-11 reacted with heat sensitive and resistant antigens, respectively. In immunofluorescence microscopy, MAbs Xco-2, Xoo-7 and Xoo-8 gave bright fluorescence. Because of the reaction patterns and the antigens recognized by MAbs Xco-2 and Xoo-7, a mixture of two MAbs can be used for rapid identification of *X. o. oryzae* using using immunofluorescence.

INTRODUCTION

Bacterial blight of rice is one of the most destructive diseases in lowland rainfed and irrigated rice in Asia and reduces yields in many rice growing areas of the world (Mew, 1989). The causal organism, *Xanthomonas oryzae* pv. *oryzae* (Swings et al., 1990), is highly variable with respect to virulence, and based on reactions on differential rice cultivars, races have been described (Horino et al., 1981; Mew et al., 1982; Mew et al., 1993; Noda and Ouchi, 1989; Ogawa, et al., 1983; Ou et al., 1971; Rehman et al., 1993). The management of bacterial blight through host resistance is difficult due to the poorly understood variability of this organism in many rice growing countries (Mew, 1987). The efforts to breed rice cultivars with durable disease resistance should be facilitated by better understanding of variability in *X. o. oryzae*.

Polyclonal antisera have been used to identify and characterize *X. o. oryzae*. Wang et al. (1981), using indirect hemagglutination, reported two serovars among 202 Chinese strains. Choi et al. (1980a) used gel diffusion and reported 3 serovars among 160 strains of *X. o. oryzae* and found that virulent and avirulent strains formed separate serovars (1980b). Addy and Dhal (1977), however, were unable to find serological differences in 45 Indian strains. Although the techniques and antisera used in these studies were not very specific, they detected serological variability in *X. o. oryzae*.

Hybridoma technology developed by Kohler and Milstein (1975) can be used to produce virtually unlimited quantities of highly specific monoclonal antibodies (MAbs). Using this technology, Benedict et al. (1989) produced three MAbs, Xco-1, Xco-2 and Xco-5 that formed four serogroups with 178 *X. o. oryzae* strains from diverse geographical locations of the world. MAb Xco-1 was pathovar specific and reacted to all of the 178 *X. o. oryzae* strains. MAb Xco-2 reacted with 87% of these strains and failed to react with only one of the 92 Philippine strains tested. In contrast to MAbs Xco-1 and

Xco-2 that reacted with strains from all geographical origins, a third MAb, Xco-5, reacted only with those *X. o. oryzae* strains that originated from the Texas and Louisiana epidemic (Jones et al., 1989) and hence recognized an antigen that is unique to strains from this region. Based on the reactivity of these MAbs, the present study was initiated to determine if serological variability existed among previously untested *X. o. oryzae* strains, representing rice growing areas of Nepal and India.

Six new serogroups that were recognized by four newly generated MAbs are reported herein. Pathogenicity tests were conducted to determine whether the serogroups formed by these MAbs were related to race and/or virulence of *X. o. oryzae* strains on rice. Antigens recognized by the new and previously reported MAbs (Benedict et al., 1989) were partially characterized. A preliminary report has been published (Rehman et al., 1992)

MATERIALS AND METHODS

Bacterial strains and cultural conditions. A list of 299 *X. o. oryzae* strains, which included 154 previously tested (Benedict et al., 1989) and 145 untested strains (140 from India and Nepal and 5 from other Asian countries), used in this study are given in Table 2.1. Eighty four strains that represented six known races from Philippines were tested for pathogenicity at the International Rice Research Institute (IRRI), the Philippines. Pathogenicity of the other strains from diverse geographical locations was tested by the providers of strains in their own areas of collection. The Nepalese and Indian strains were characterized at the University of Hawaii on the IRRI standard set of differential rice cultivars (Rehman et al., 1993). The 106 other xanthomonads and 61 strains from the other plant pathogenic and saprophytic bacterial genera and species used to test antibody specificity are shown in Table 2.2. For screening, strains stored at -20 C were first streaked on PSA (Tsuchiya et al., 1982) and were transferred to YGA (Alvarez

et al., 1978). They were incubated at 28 C for 48 to 72 hr and harvested in 0.5% formalin containing phosphate-buffered saline (PBS) (NaCl, 8.0 g, Na_2HPO_4, 1.15 g and K_2HPO_4, 0.2 g per liter; pH 7.4) and stored at 4 C.

Enzyme-linked immunosorbent assay (ELISA). The formalin treated cells of the bacterial strains were washed three times in PBS, resuspended in 0.05 M carbonate-bicarbonate buffer (pH 9.6), and the suspensions were spectrophotometerically adjusted to OD A_{600} = 0.1. These suspensions were coated at 0.1 ml per well onto 96 well polyvinyl chloride microtiter plates (Dynatech Labs., Inc., Alexandria, VA and Costar, Cambridge, MA) and were dried at 37 C overnight in a circulating air incubator. Plates were covered with transparent plastic tape after drying and stored at 4 C until use. For ELISA, plates were blocked with 5% skim milk in PBS (Johnson et al., 1984) for 15 min. and washed once with 0.16 M borate buffer (pH 8.3). Each of the following reagents was diluted 1:1000 in a 1:3 dilution of 5% skim milk in PBS (i) MAb (ii) rabbit anti-mouse antibody, and (iii) Protein-A-horseradish peroxidase (Bio-Rad Labs, Richmond, CA). One tenth ml of each reagent was added per well and incubated for 60 min. Each step was followed by 4 washes in borate buffer at room temperature. The substrate, 5-amino-salicylic acid (0.05%), and 0.006% H_2O_2 in phosphate-EDTA buffer was then added, and the absorbance was measured after 60 min with a Titertek Multiskan plate reader at $A_{450nm.}$

Monoclonal antibody production. The procedural details for production of MAbs are given elsewhere (Alvarez et al., 1985; Benedict et al., 1989). Bacterial cells were always washed 3X in PBS for preparing antigens to immunize BALB/c mice twice intraperitonealy at 14 day intervals, either with formalin treated or live whole bacterial cells. Immunizations were made using individual strains or mixtures of different strains of *X. o. oryzae*. For some immunizations bacterial cells were first disrupted in a French pressure cell (FPC) at a pressure of 24,000 psi or sonicated in a Biosonik model II apparatus (Bronwill Scientific, Rochester, NY) with 4 or 10 mm titanium probe and were

injected into mice before or after centrifugation. A total of 14 fusions were made following immunizations with different preparations of the antigens. For the first two fusions, formalin-killed whole bacterial cells of *X. o. oryzae* strains PXO85 (race 1) and PXO63 (race 2) were used separately as antigens. The next fusion was made using a FPC extract of PXO35 (race 1) as an immunogen. In five more fusions, FPC extracts of PXO61 (race 1) were used to immunize mice either directly or after precipitating with 50% saturated ammonium sulfate. Freund's incomplete adjuvant was always used for the first immunization with FPC preparations. For two other fusions, one was made by immunizing mice with a sonicated preparation of *X. o. oryzae* strains, PXO35 (race 1), PXO63 (race 2), PXO86 (race 2), PXO79 (race 3), PXO70 (race 4), PXO105 (race 5), PXO99 (race 6), representing six known Philippine races (mixed in equal proportions) and the other was made by using sonicated strain PXO61 (race 1) as the antigen. Another fusion was made in which formalin killed bacterial cells of an Indian strain T2 were used as the antigen. The last three fusions were made by immunizing mice with live and formalin-killed whole cells (mixed in 1:1 ratio) of each of the Nepalese *X. o. oryzae* strains NXO215, 261, and 263, respectively. Fusions were made by mixing spleen cells of immunized mice with P3-X63 myeloma cells, as described previously (Alvarez et al., 1985) and ELISA was used for initial screening of antibody-producing supernatant fluids against the selected strains of *X. o. oryzae*. The *Pantoea* (*Erwinia*) *herbicola* strain, Eh-1, was used as a negative control. The desirable hybridomas were expanded, screened for specificity against the strains given in Table 2.1 and 2.2 and were cloned twice by limiting dilution before injecting into mice for ascities production. Ascitic fluids were cleared by centrifugation and retested against the strains listed in Tables 2.1 and 2.2.

Antibody titers were determined by developing binding curves using two fold dilutions of MAbs against their homologous strains in duplicate in ELISA. *Pantoea (Erwinia) herbicola* strain Eh-1 was used as a negative control. Antibody isotypes were

determined in Ouchterlony double diffusion assays (Goding, 1986) using anti-mouse isotype antisera (Litton Bionetics Inc., Kensington, MD) against the antigens prepared from concentrated culture supernatants of hybridoma cells.

Characterization of antigens. Bacterial lipopolysaccharide (LPS) of *X. o. oryzae* strains, X1-5, PXO35, PXO86, NXO215 and NXO261 were extracted from formalin killed bacteria using the hot phenol method described by Westphal and Jann (1965). The LPS-containing fractions were dialyzed against distilled water at 4 C to remove phenol and were concentrated under reduced pressure. RNase (Sigma Chemical Co., St. Louis, MO) and Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) treatments were given to LPS preparations and were centrifuged at 80,000 g for 6 hr. Pellets thus obtained were dissolved in distilled water and stored at -20 C in small aliquots.

For the study of the antigen recognized by MAb Xco-1 (Benedict et al., 1989), FPC or sonicated preparations were made, as described above, from heavy bacterial suspensions of *X. o. oryzae* strain X1-5 in PBS. Supernatants from these preparations were concentrated as above and stored at -20 C. These preparations also were given enzyme treatments with proteinase K, pronase E, trypsan, pepsin, papain or RNase. The FPC preparations also were used in heat sensitivity experiments in which they were heated at 100 C for 3 min either in PBS or in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Laemmli 1970) or were heated in hot water baths set at 50, 60, 70, 80, 90 and 100 C. In some experiments bacterial suspensions in PBS, adjusted to OD A_{600} = 0.4 or more, were heated in Eppendorff tubes in a boiling water bath for 15, 30, 45 and 60 min, and centrifuged and the supernatants were saved.

All these antigen preparations were tested in ELISA, Ouchterlony double diffusion assays or by the SDS-PAGE western blotting technique (Towbin et al., 1979; Alvarez et al., 1991) with adequte controls. The LPS, sonicated, FPC or heated preparations were mixed 1:1 in a 2X sample buffer (Laemmli 1970) with or without 5% 2-mercaptoethanol.

Electrophoresis was routinely performed in 5% stacking and 7.5% separating gels. Occasionally 6 to 15% separating gels also were used. The Coomassie blue and silver staining of polyacrylamide gels for Xco-1 and Xco-5 antigens was as described (Morrissey, 1981), and silver staining of Tsai and Frasch (1982) was used to detect LPS. Procedures for western blotting of the gels with ^{125}I-protein-A and molecular weight markers were the same as described by Alvarez et al. (1991). A non-radioactive detection system (ECL) and rainbow molecular weight markers, with M_r range of 14,300 to 200,000, also were used for western blotting following manufacturer's instructions (Amersham Life Science, IL).

Immunofluorescence microscopy (IF) and immunofluorescence colony staining (IFC). Bacterial suspensions were washed twice with PBS, heat fixed and air dried on glass slides at room temperature. Slides were then placed in acetone for 10 min, washed in PBS, and treated with MAbs at different dilutions for 60 min. After washing 3 to 4 times with PBS, the rabbit anti-mouse immunoglobulin conjugated with fluorecein isothyocyanate-FITC (Miles Scientific, Napeville, IL) was added and incubated in the dark for 60 min. Slides were washed thoroughly and examined under UV light (490 nm) with an epifluorescence microscope after mounting in a 9:1 glycerol-CBC(2X) mounting medium.

A modified IFC procedure (Van Vuurde, 1987; Mochizuki et al., 1992) was used with modifications. Briefly, pure cultures of *X. o. oryzae* strains were grown for 24 to 48 hr, adjusted to $OD_{A600} = 0.1$ in PBS, and 10-fold dilutions were made. The sterilized PSA medium with 1.2% agar was cooled to approximately 37 to 40 C. Bacterial suspensions (0.1 ml) from 10^{-2} to 10^{-4} dilutions were placed in the middle of a 90 mm Petri plate and mixed with about 12 ml of PSA by swirling the plate quickly to avoid solidification. Plates were incubated at 28 C for about 24 to 72 hr or until pinhead sized colonies were visible at 40X under the dark field illumination of a stereomicroscope. Small agar pieces

(approximately 1 cm^2) containing these colonies were cut and dried at 37 C in a convection oven. These pieces were rehydrated with 1:50 or 1:100 dilutions of the MAbs Xoo-7, Xoo-8 and a previously reported MAb Xco-2 (Benedict et al., 1989). Plates were covered with aluminum foil and kept at room temperature for about 4 to 12 hr on a rotary shaker adjusted to 200 rpm. Agar pieces were washed 3X with PBS and stained with rabbit anti-mouse-FITC immunoglobulin, as above, for 4 hr. They were viewed under the UV light after washing 3X in PBS and mounting in glycerol-CBC buffer as above. Colonies that gave bright yellow green light were considered positive.

Immunoelectron microscopy. Procedures of Robinson et al. (1984) and Alvarez et al. (1991) were modified. Sterile distilled water was used for making bacterial suspensions and washing the carbon stabilized Formvar coated grids (Ted Pella, Inc., Tustin, CA). The goat anti-mouse antiserum conjugated with 5-nm gold spheres (Sigma Chemical Co., St. Louis, MO) were used after grids were incubated with MAbs Xoo-7, Xoo-8, Xoo-10 and Xoo-11. Grids were stained with 1% uranyl acetate for 2 min in the dark and then were washed, dried and examined with a transmission electron microscope operated at 60 kV.

Pathogenicity studies. Seeds of rice cultivar IR-1545-339 were planted in sterile super soil mixed with slow release 14:14:14 (N-P-K) osmocote granular fertilizer in 10 cm diameter pots (2 seeds per pot). Plants were kept in a greenhouse for 5 weeks prior to inoculation. Strains were grown on PSA for 24 to 48 hr at 28 C in an incubator. Bacterial suspensions were made in sterile PBS, and inoculum concentrations were adjusted to OD $A_{600} = 0.2$ in a spectrophotometer. Inoculum for each treatment was prepared 1 to 2 min before inoculation. The scissor-clipping method described by Kauffman et al. (1973) was used for inoculation. All the leaves on two plants per pot were clipped individually after dipping sterile scissors into inoculum (eight plants per treatment). The experiment was repeated with four plants per treatment. Plants were covered with plastic bags and kept at

room temperature overnight. The next day, bags were removed and pots were submerged in water to soil level in deep plastic containers and moved into a growth chamber, adjusted to 14 hr light and 10 hr dark cycles with temperatures at 28 C and 24 C, respectively, and relative humidity of 75%. For negative controls, plants were cut with scissors dipped in sterile PBS. Lesion lengths were recorded 14 days after inoculation (Koch and Mew, 1991). Average lesion lengths for each strain were used to assign resistant (<10 cm) or susceptible (>10 cm) reactions. Bacterial streaming was used to confirm the presence of *X. o. oryzae* in rice leaves. Bacterial ooze from diseased leaf tissues also was streaked on PSA from representative samples and tested by ELISA to confirm identity of the inoculated strain.

RESULTS

Monoclonal antibody production. Approximately 2800 hybridomas were produced from a total of 14 fusions of myeloma and mouse spleen cells following immunization with *X. o. oryzae* strains (Table 2.3). Irrespective of the antigen preparations used to immunize mice, two major specificity patterns similar to MAbs Xco-1 and Xco-2 (Benedict et al., 1989) were repeatedly observed when the parent hybridomas and first clones were tested with *X. o. oryzae* strains. None of these clones were specific for race(s) of *X. o. oryzae* from the Philippines, India and Nepal. Nonetheless, specificities different from those mentioned above were observed, and the clones that gave high binding titers and remained stable through double cloning were selected for further study. Five such clones: 211-G4, 240-40, 226-42, 239-11 and 232-D20 were selected and designated as MAbs Xoo-7, Xoo-8, Xoo-9, Xoo-10, and Xoo-11, respectively.

The binding curves of MAbs Xoo-7 (IgG3k), Xoo-8 (IgG3k), Xoo-9 ($IgG2_ak$), Xoo-10 (IgG3k) and Xoo-11 (IgM) are shown in Figure 2.1. MAb Xoo-11 gave ELISA absorbance values of 1.2 and above with many *X. o. oryzae* strains and lower values

between (0.2 to 0.4) with a few *X. o. oryzae* strains. Similarly, MAb Xoo-10 showed quantitative differences among strains. Titers of MAbs Xoo-7 and Xoo-8 observed in EILISA were relatively higher than MAbs Xoo-9, Xoo-10 and Xoo-11. Based on the replicate determinations of binding curves, 1:1000 dilutions of these antibodies were used routinely. In order to delineate positive and negative ELISA reactions, the ELISA absorbance values of a negative control, *P. herbicola* strain Eh-1 (Fig. 2.1) were subtracted from the values of *X. o. oryzae* strains and the derived values of ≥ 0.1 were considered positive. The ELISA values of these selected MAbs with the strain Eh-1 usually were below 0.1.

Antibody Xoo-7 reacted with 33 *X. o. oryzae* strains that included 16 strains from Nepal, 15 from India, and one each from the Philippines and Colombia. Ninety two percent of *X. o. oryzae* strains tested in this study reacted either with MAb Xco-2 or with MAb Xoo-7 (Fig. 2.2) and the remaining 8%, that included mostly the atypical strains, were negative with both MAbs. None of the 299 strains tested so far, have reacted with both MAbs. With a few exceptions, all *X. o. oryzae* strains that had strong positive reactions (OD $A_{450} > 1.0$) with MAb Xoo-7 had low reactions (OD $A_{450} < 0.5$) with a pathovar-specific MAb Xco-1 (Benedict et al., 1989). MAb Xoo-8, reacted with the same 33 strains that reacted with the MAb Xoo-7 and in addition reacted with two weakly virulent Indian strains, T1 and T2 (Gnanamanickam et al., 1993). This MAb also reacted with 3 strains of *X. campestris pv. citri*, two strains of *X. c.* pv. *manihotis* and two strains of *Xanthomonas* sp from ti (*Cordyline terminalis*). MAb Xoo-9, however, reacted exclusively with two weakly virulent Indian strains, T1 and T2. MAb Xoo-10 reacted with 15 strains that included 10 from Nepal, three from India, and one each from the Philippines and Taiwan. MAbs Xoo-7, Xoo-9 and Xoo-10 did not react with any of 106 xanthomonads or 61 non-xanthomonads of various genera and species of phytobacteria. MAb Xoo-11 reacted with 25 strains that included nine strains each from India and the

Philippines, three from Nepal, one each from Bangladesh, China, Indonesia and Taiwan. This antibody also reacted with three strains of *X. campestris* pv. *citri*, one each of pv. *dieffenbachiae* and pv. *begoniae*, two strains each of pv. *manihotis* and pv. *syngonii* and two strains of *Xanthomonas sp.* from ti (*Cordyline terminalis*).

Based on the reactions of these MAbs with 299 *X. o. oryzae* strains, six new serogroups, namely IIb, VI, V, VII, VIII, and IX, were formed (Table 2.4). Serogroup IIb emerged from the subdivision of a previously reported serogroup II (Benedict et al., 1989). Serogroup IIa comprised six strains that did not react with any of the four new MAbs, whereas serogroup IIb contained 30 strains (14 Nepalese, 14 Indian, one Philippine and one Colombian strain) that reacted with new MAbs Xoo-7 and Xoo-8 in addition to MAbs Xco-1. Serogroup V comprised two weakly virulent strains T1 and T2. Serogroup VI contained 23 strains that included nine each from India and the Philippines, two from Nepal and one strain each from China, Bangladesh and Indonesia. Serogroup VII comprised two strains, one each from Taiwan and Nepal. Group VIII was composed of 12 strains that included eight strains from Nepal, two from India and one each from the Philippines and Taiwan. Group IX contained one Indian and two Nepalese strains.

Characterization of the antigens. Phenol extracts of strain X1-5, either untreated or treated with proteinase K, pronase or RNase did not contain antigen precipitable with MAb Xco-1 in immunodiffusion. MAb Xco-1, however, detected a heat sensitive, high molecular weight antigen with at least 2 to 3 major bands appearing between M_r 96,000 to 150,000 when unheated, unreduced FPC extracts were tested in western blots (Fig 2.3A). The antigen was partially digestible with proteinase K and other proteolytic enzymes. Thus, its electrophoretic mobility changed after digestion with these enzymes. After digestion, the molecular weight of the antigen ranged between M_r 93,000 to 123,000, with a new component appearing at M_r 61,000 (Fig. 2.3A). Similar banding patterns were observed when FPC extracts were digested with enzyme pronase E, pronase plus

proteinase K and RNase plus proteinase K (Fig. 2.3B). Digestion with enzymes papain, trypsin and pepsin also produced similar results. Reduction with 5% 2-mercaptoethanol did not affect the mobility or binding of Xco-1 with the antigen. A thick band similar to one observed in western blots of enzyme treated FPC extract in Figure 2.3 also was visible with Coomassie blue and silver staining on the gels containing different dilutions of enzyme digested FPC extracts (Fig.2.4). When FPC extracts were heated in PBS, binding of MAb Xco-1 with the antigen decreased gradually up to 90 C (Fig. 2.5A) and was almost completely lost at 100 C (Fig. 2.5B). On the other hand, the activity of the antigen was completely lost at 50 C when heated in sample buffer containing SDS (Fig. 2.5B).

Both FPC and phenol extracts of *X. o. oryzae* strain, X1-5, treated with enzymes proteinase K, pronase E and RNase, contained antigens that precipitated with MAb Xco-2 in immunodiffusion. The antigen also was detected in western blots of FPC extracts treated with enzymes (Fig 2.6A). The hot phenol extracts of strains XI-5 and PXO86, untreated or enzyme treated and ultra-centrifuged, formed a ladder like patterns typical of LPS in the silver stained gels (Fig. 2.6B) and the same banding patterns were detected by MAb Xco-2 in the corresponding western blots (Fig. 2.6C).

The antigen recognized by MAb Xco-5 was found heat sensitive previously (Benedict et al., 1989) and in the present study. It was partially sensitive to enzyme proteinase K as was the Xco-1 antigen. A thick band of a high molecular weight similar to Xco-1 antigen was visible in western blots of FPC extract of strain X1-5 only after digestion with proteinase K. Repeated attempts to visualize undigested antigen in western blots have not been successful so far (data not shown).

The antigen in hot phenol extracts of strain PXO35 precipitated in immunodiffusion tests with MAbs Xoo-7 and Xoo-8. The untreated or proteinase K treated/ultracentrifuged hot phenol extracts formed bands in a ladder similar to the bands of purified LPS of *Salmonella typhosa* in a silver stained gel (Fig 2.7A). In the

corresponding western blots, the same bands were detected by MAb Xoo-7 (Fig 2.7B) and MAb Xoo-8 reacted with PXO35 (Fig. 2.7C) whereas *Salmonella typhosa* did not react with either MAb.

The antigen detected by MAb Xoo-10 was heat sensitive. Because binding with MAb Xoo-10 was completely lost when bacterial cells of strain NXO261 were heated in PBS at 100 C for 15 to 60 min and tested in ELISA (Fig. 2.8). Similarly, heated bacterial cells also failed to bind with Xco-1 as shown by ELISA (Fig. 2.8).

The antigen detected by MAb Xoo-11 was heat resistant. Binding remained unaffected in ELISA even when bacterial cells were boiled up to 1 h in PBS (Fig. 2.8). When the same heated cells were tested in western blots of 7.5% SDS-PAGE gels, this antigen also was detected by Xoo-11 (Fig. 2.9A). No bands were detected from the lanes containing unheated bacterial cells (Fig 2.9A). Supernatant obtained after centrifugation of the heated cells also gave no bands. In a 15% gel, sonicated and heated extracts of NXO215 formed three major bands and few minor bands (Fig. 2.9B). The molecular weights of these bands ranged between M_r 21,000 to 46,000 as observed in 12.5% SDS-PAGE gel (data not shown).

Immunofluorescence microscopy and immunofluorescence colony staining. MAbs Xoo-7 and Xoo-8 gave bright fluorescence when tested with *X. o. oryzae* strain PXO35 in an indirect IF, whereas MAbs Xoo-10 and Xoo-11 failed to fluoresce when tested against homologous strains NXO261 and NXO215, respectively.

Results of indirect IFC were similar to those observed in IF for MAb Xoo-7 and Xoo-8. Colonies of PXO35 gave bright fluorescence after 48 hr of growth in PSA with MAb Xoo-7 (Fig. 2.10A) and MAb Xoo-8 (Fig. 2.10B). MAb Xco-2 also gave bright fluorescence when tested with PXO86 in IFC. Large fluorescent halos were observed around the colonies if colonies were grown for 72 to 96 hr. Since MAbs Xoo-10 and Xoo-11 failed to fluoresce in IF, they were not tested by IFC.

Immunoelectron microscopy. Abundant decoration with gold particles was observed by immunogold electron microscopy on bacterial cell surfaces of strain PXO35 reacted with MAb Xoo-7 (Fig. 2.11A) and MAb Xoo-8 (Fig. 2.11B). The distribution of antigens detected with both MAbs was consistent with heat stable LPS antigens, as reported previously for MAb Xco-2 (Benedict et al., 1989). The strain NXO215 also was decorated with gold particles but to a lesser extent than the MAbs Xoo-7 and Xoo-8 (Fig. 2.11D). However, no gold particles were found on strain NXO261 that was reacted with MAb Xoo-10 (Fig. 2.11C). The characteristics of the MAbs made to *X. o. oryzae* and their respective antigens are summarized in Table 2.5.

Pathogenicity studies. Two *X. o. oryzae* strains were selected for inoculation from each of the 10 serogroups to determine whether the serogroups related to the virulence of these strains on rice. Serogroups III, IV, and V contained low virulence strains from the U.S.A. and India but there was no other obvious relationship between serogroups and virulence (Fig. 2.12). The serogroups showed no relationship to the race designations of strains from the Philippine, India, or Nepal.

DISCUSSION

Most of 299 *X. o. oryzae* strains representing diverse rice growing areas of the world belonged to serogroup I and reacted with previously reported MAbs Xco-1 and Xco-2 (Benedict et al., 1989). Antibodies with similar specificities were repeatedly generated in the present study following immunizations of mice with numerous different cell preparations. Several new antibodies also were generated, however, and differential reactions with the MAbs resulted in the designation of six new serogroups. Group IIb, V and IX contained predominantly strains from India and Nepal that reacted with new MAbs Xoo-7, Xoo-8 and Xoo-9. Although MAbs Xoo-10 and Xoo-11 reacted to a relatively

small number of strains, they also identified serogroups that predominantly contained strains from India and Nepal.

Another observation made in this study was that a MAb of identical specificity to Xoo-7 was repeatedly generated when a Philippine strain PXO35 (race 1) was used to immunize mice either individually or as mixture with other strains. This was the only strain among the 92 Philippine strains of *X. o. oryzae* that did not react with MAb Xco-2 (Benedict et al., 1989). In RFLP analysis of 98 Philippine *X. o. oryzae* strains, PXO35 also was the only strain with a unique RFLP pattern and was distantly related (63% similarity) to other *X. o. oryzae* strains by cluster analysis of the RFLP banding patterns (Leach et al., 1992). The fact that MAb Xoo-7 reacted with 31 *X. o. oryzae* strains from Nepal and India in addition to strain PXO35 from the Philippines and one strain from Colombia, indicated that the serologically distinct, "PXO35 type-population" is prevalent in India and Nepal.

The *X. o. oryzae* strains from India and Nepal were highly virulent on most cultivars containing resistance genes that are presently used for rice breeding at IRRI as compared to the strains from other Asian countries (Adhikari, et al., 1994; Adhikari et al., 1993; Gupta, et al., 1986). Moreover, RFLP analysis of *X. o. oryzae* strains from Asian countries also revealed a high degree of polymorphism in Nepalese and Indian strains compared to the strains from China, Indonesia, Malaysia, Korea and the Philippines. Thus they formed a separate cluster (5) which makes Indian and Nepalese strains distinct, whereas strains from other countries formed 4 clusters based on their geographical origin (Adhikari et al., 1993). Cluster 5, however, also contained a few strains from other countries, as did the predominantly Indian/Nepalese serogroups defined in this study. Clustering of these few strains with Indian and Nepalese strains was considered to be due to pathogen migration perhaps during international exchange of contaminated germ plasm (Adhikari et al., 1993).

Like MAb Xco-5, that reacted with only those *X. o. oryzae* strains that were collected in the U.S.A. (Benedict et al., 1989; Jones et al., 1989), the five new MAbs reacted to groups of strains that were predominantly found in India and Nepal, which were sub-divided into six new serogroups. The strains from other countries that reacted to the MAbs Xoo-7, Xoo-8, Xoo-10 and Xoo-11, may have been a result of pathogen migration as suggested by Adhikari et al. (1993).

The weakly virulent strains T1 and T2, associated with rice in India (Gnanamanickam et al, 1993), were the only two strains that failed to react with pathovar specific MAb Xco-1, and formed a separate serogroup, V that comprised only these two strains. These strains shared some phenotypic characters with both *X. o. oryzae* and the bacterial leaf streak organism, *X. o. oryzicola* and had several phenotypic responses similar to the low virulence U.S. (Texas) strain XI-5; nevertheless they were clearly different from the U.S.A. strains as well as *X. o. oryzae* and *X. o. oryzicola* by RFLP analysis (Gnanamanickam et al, 1993). Although strains T1 and T2 had a unique antigen that bound exclusively with MAb Xoo-9, they also shared a common antigen with 33 *X. o. oryzae* strains from India, Nepal, the Philippines, and Colombia, detected by MAb Xoo-8 that was generated when a strain NXO263 from Nepal was used as immunogen. On the other hand, a clone that produced an antibody of identical specificity to MAb Xoo-8, also was obtained when strain T2 was used as immunogen. Thus, the MAbs Xoo-8 and Xoo-9 clearly indicated that T-strains are antigenically related to *X. o. oryzae*. The significance of these two strains as pathogens of rice is unclear at present. They may represent an evolutionary phase between *X. o. oryzae* or *X. o. oryzicola* because they shared some phenotypic features with both pathovars or may belong to a completely different pathovar that have become associated with rice (Gnanamanickam et al., 1993). These strains need to be monitored carefully for changes in their virulence to rice.

The pathovar specific MAb Xco-1 detected a common antigen found on 297 *X. o. oryzae* strains in this and a previous study (Benedict et al., 1989). Based on the criteria for determining the nature of an antigen (Goding, 1986), the antigen detected with the MAb Xco-1 was considered a protein moiety because of its denaturation with SDS, heat sensitivity and partial digestibility with proteolytic enzymes. An antigen with similar characteristics also was detected with a MAb C1 specific for a *X. c. citri* strain XC70 (Alvarez et al., 1991). The heat stable antigen recognized by MAb Xco-2 was identified as LPS here and previously (Benedict et al., 1989) because hot phenol extracts (Westphal and Jann, 1965) of *X. o. oryzae* strains precipitated in immunodiffusion, and "ladder-like" patterns, typical of LPS, were observed in silver stained gels and western blots of hot phenol and heat extracts, treated with RNase and proteolytic enzymes and sedimented at 80,000 g in an ultra-centrifuge. Based on these criteria, the antigens recognized by MAbs Xoo-7 and Xoo-8 also were identified as LPS. They too produced a ladder of bands similar to purified LPS of *Salmonella typhosa.* The LPS antigen detected by MAb Xoo-8 however, is also found on *X. campestris pv. citri*, pv. *manihotis* and *Xanthomonas sp.* from ti (*Cordyline terminalis*). Likewise the antigen of MAb Xoo-11 was shared with these pathovars, in addition to *X. c.* pv. *dieffenbachiae*, pv. *begoniae,* and pv. *syngonii.* Such sharing of the antigens with *X. campestris pv. citri*, pv. *manihotis* and *Xanthomonas sp.* from *ti* (*Cordyline terminalis*) also was found previously (Alvarez et al., 1991), the significance of which is not known at present.

Since MAb Xco-1 appears to detect a unique antigen, it is useful for identification of all the strains *X. o. oryzae*; however, the antigen detected is a protein moiety and weak reactions may occur by ELISA (Gnanamanickam et al., 1993). Moreover, a weak reaction occurs with IF (Benedict et al., 1989), therefore, this MAb is not preferred for detection by IFC. MAbs that detect LPS antigens are the best choices because reactions are generally high, and LPS is heat stable. MAbs Xco-2, Xoo-7 and Xoo-8 can be used with

the IFC technique because they detect LPS antigens, belong to immunoglobulin class IgG (with better diffusion through agar than larger IgM molecules) and gave bright fluorescence. Because of the high titers and reaction patterns observed in ELISA, MAbs Xco-2 and Xoo-7 can be mixed together as a "cocktail" for rapid detection and identification of the most prevalent strains of *X. o. oryzae.*

Polyclonal antisera, that related serotype to virulence of *X. o. oryzae* were reported (Choi et al., 1980b; Lin et al 1969). Likewise, in the present study, serogroups III, IV and V (Benedict et al., 1989; Rehman et al., 1992) included only strains of low virulence, but the strains in these groups are considered atypical *X. o. oryzae* on the basis phenotypic characteristics, RFLP analysis and symptoms produced on susceptible rice cultivars (Jones et al., 1989; Gonzalez, 1991; Gnanamanickam et al., 1993, Rehman et al., 1992). It is possible that the polyclonal antisera that related serogroups to virulence also reacted with some atypical *X. o. oryzae* isolated from rice. Association of fast growing, low virulence atypical strains with rice may be partially responsible for the problems that are faced in detection and identification of typical, slow growing *X. o. oryzae.* They may also play a role in the controversy regarding the seed transmission of *X. o. oryzae* because they can be mistakenly identified as typical strains and result in the wrong conclusions. Based on the information gained from the present and previous studies, the epidemiological significance of atypical strains as *X. o. oryzae* should be re-evaluated.

None of the MAbs produced in this study or previous studies were specific for races of this pathogen described from the Philippines, Nepal and India (Benedict et al., 1989; Gnanamanickam et al., 1993). Despite serological, genetic (Leach et al., 1992) or physiological and biochemical (Vera Cruz et al., 1984) variability in *X. o. oryzae,* no group of tests or probes have been found that exclusively identify races and thus replace the time-consuming method of studying variability of this organism by pathogenicity tests on rice cultivars (Mew, et al., 1993). Nevertheless, use of new and previously reported MAbs

(Benedict et al., 1989) and DNA probes (Leach et al., 1992) has a definite advantage for detection, identification and monitoring sub-groups of *X. o. oryzae* beacuse of their speed, specificity, sensitivity These MAbs permit development of efficient, sensitive and reliable detection and identification methods for *X. o. oryzae*.

Table 2.1. *Xanthomonas oryzae* pv. *oryzae* strains used to test monoclonal antibodies

Origin		Strain designations	No. of strains tested	Source
Nepal		NXO: 101, 117, 142, 147, 149, 151, 156, 157, 159, 160, 161, 174, 180, 181, 194, 195, 196, 198, 200, 201, 207, 212, 215, 216, 228, 237, 239, 240, 245, 256, 259, 260, 261, 263, 275, 282, 331, 334, 340, 347, 354, 355, 356, 357	44	1
India		G: 1, 2, 3, 4, 5, 7, 8, 10, 13, 14, 15; GWB: 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18; AP: 10, 11, 19, 23, 24, 25, 27, 28, 28-2, 29, 32, 33, 35, 36, 38-2, 40; M: 1, 1X, 3, 7, 10, 11, 12, 13, 15, 16, 17, 21, 22, 24, 31, ADT19, ADT20, T1, T2, GPKL-50	64	3
		8201M-8206M, 8208M-8214M, 8217M-8220M, 8223M, 8225M-8228M, 8308M-8309M, 8311M, 8314M, 8318M-8319M, 8322M-8324M, 8327M	32	8
Philippines	Race 1:	PXO: 1, 4, 5, 9, 10, 13, 14, 15, 19, 20, 23, 32, 34, 35, 38, 48, 52, 61, 85, 151	20	6
Philippines	Race 2:	PXO: 63, 78, 82, 83, 86, 103, 104, 126, 135, 138, 139, 140, 158	13	6
Philippines	Race 3:	PXO: 79, 81L, 87, 88, 141, 146, 147, 148, 152, 153, 154, 155, 156, 160	14	6
Philippines	Race 4:	PXO: 69, 70, 71, 113, 125, 129	6	6
Philippines	Race 5:	PXO: 45, 80, 105, 106, 107, 108, 109, 110, 111, 112, 112A, 130,144, 145, 150	15	6
Philippines	Race 6:	PXO: 99, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 127, 128	14	6

Race 0:	PXO: 40, 101	2	6
Australia(AU), Bangladesh(B), India(H), Indonesia(IG), Japan(N), Taiwan(TW), Thailand(TL)	AU6, B13, B8, B77, BU1, BU2, BU6, BU10, BU12, BU14, BU15, BU20, BU27, CL4-4, CL6, H14, H66, H100, H200, H201A, IG23, IG24, N1P15, TL23,	25	2
Colombia	CIAT 1185, 1186	2	9
Australia, Bangladesh, China, India, Taiwan, Thailand	FXO (race unknown): 35, 36, 39, 40, 50, 60, 61, 63, 64, 65	10	6
Taiwan	IRN 395, XO: 3, 16, 20b, 24	5	2
Texas	X1-5, X1-6, X1-7, X1-8, X1-10, X4-1B, X4-1C, X4-2C, X4-4C, X4-3D, X4-4D, X4-8C, X7-5A, X7-2D, X7-3E, X11-1A, X11-5A, X11-1B, X11-4B, X11-5B, X11-6B, X11-2D, X13-3A, X13-5C, X13-2E	25	4,5
Louisiana	X8-1A, X8-1B, RU87-17, RU87-18	4	4,5
Japan	JXO: T7133, T7174	2	5,7
	A1779, A1804	2	2

1, T. Adhikari, Nepal; 2, I. W. Buddenhagen, California; 3, S. Gnanamanickam, India; 4, C. F. Gonzalez, Texas; 5, J. E. Leach, Kansas; 6, T. W. Mew, Philippines; 7, S. S. Wakimoto, Japan; 8, T. Yamamoto, Japan; 9, R. S. Zeigler, Colombia.

Table 2.2. Strains of various bacterial genera used to test the specificity of monoclonal antibodies.

Genus/species or pathovar	No. of strains tested	Strain designation	Source
Xanthomnas albilineans	3	G7, HV5, R8	4
X. campestris			
pv. *alfalfae*	1	G22	14
pv. *armoraciae*	4	756, XLS-2, G3-27, XLS-4, 417,	1, 5,30
pv. *begoniae*	3	A915, X45, QR30	1, 7, 28
pv. *campestris*	4	A249, A902, EEXC114, RR68	1, 12, 31
pv. *carotae*	4	ARCO-1, ARCO-2, ARCO-3, B 75	23, 30
pv. *citri*	7	XC59, XC62, XC63, XC64, XC69, XC70, XC90	9
pv. *dieffenbachiae*	5	D78-3, D110, D147, D194, D238,	1,21
pv. *euphorbiae*	2	A912-2, A875-1	1
pv. *hederae*	4	X17, X24, X25, X261	7
pv. *malvacearum*	4	X27, X108, X203, X204	7
pv. *manihotis*	4	HMB9, HMB38, HMB286, HMB296	19
pv. *pelargonii*	5	X38, X127, IPO272, IPO273, M62	7, 28, 20
pv. *phaseoli*	4	A602-11, BXP118, BXP18, M191	1, 11, 6
pv. *oryzicola*	12	A2709, A2715, S5, S14, S25, S35, S41, S44, S57, S63, S67, S70	18, 29
pv. *raphani*	5	86-1, 86-2, 86-3, X85-F, X85-AY	15
pv. *syngonii*	4	X159, X162, X163, X181	7
pv. *translucens*	5	MO1, MO3, MO5, MO6, MO7	24
pv. *urticae*	1	012	7
pv. *vesicatoria*	5	X297, XCV-1, XCV-2, Fukuyama Kahuku, 32046-84, Kawaii I Uni Chi	1, 10
pv. *vitians*	3	A674-2B, X42, QR71	1, 17, 8
X. campestris (undescribed pathovars) from			
Allium cepa (onion)	6	A88-3, A226-3, B3, A3285, A6(8), A255-4	1
Cordyline terminalis (ti)	2	A910-2, A910-3	1
Cynodon dactylon (Bermuda grass)	4	C130-2A, C130-2AR, C210-2, C260-4A	1
Polycias gulifoylei (Panax)	1	G175	1
X. maltophilia	4	DR34, W46, DR52, DR54	1
P. avenae	2	BCE 12, CHY9 NF	32
Pseudomonas cattleyae	1	P24	7

P. cepacia	1	PC25 (QR34)	27
P. chicorii	5	P135, P111, C546-2, C606-2, C397-1	1,7
P. fluorescens	2	GUAT 1388-2, B1034-2	32
P. fuscovaginae	3	B1621-3, N1012-1, SR532	32
P. marginalis	2	P10, ATCC10844	3, 7
P. solanacearum	3	A3295, QR87(B1), QR86(K60)	26
P. syringae			
pv. *syringae*	5	PSS-1, PSS -2, PSS-3, PD290, ATCC19310	1, 3, 10
pv. *phaseolicola*	5	A798-2, G50 Tt, HB 20, PD 270 , PD 572	1, 16
pv. *putida*	2	S15-2 IR, B1106-10	32
pv. *tabaci*	2	P13, QR69	7, 22
pv. *tomato*	1	PST-102	10
P. viridiflava	1	PV-1	10
P. viridilivida	1	ATCC19048	3
Erwinia atroseptica	4	PD738, A3254, EA9, EA7	13, 16
E. carotovora	3	ECC1, ECC7, UC 176	13, 27
E. chrysanthemi	2	A1073, A3312	19, 21
E. herbicola	1	Eh-1	1
Agrobacterium radiobacter	1	A414	1
A. rhizogenes	1	TR108	27
A. tumefaciens	1	UCBPP 388	25
Corynebacterium flaccumfaciens	1	ATCC 6887 (QR79)	3
C. poinsettiae	1	ATCC 9682 (QR78)	3
Clavibacter fasciens	1	ATCC 12975 (QR81a)	3
C. insidosum	1	ATCC 10253 (QR80)	3
C. michiganense	2	A518-1, B100,	1, 30
C. rathayi	1	ATCC13659 (QR82)	3
C. sepedonicum	3	ATCC9850(QR77), R8, A2044	2, 3
E. cloacae	2	F2, YP-16	21

1, Local isolation; 2, Agdia, IN; 3, American Type Culture Collection; 4, P. Baudin, France 5, L. C. Black, LA; 6, I. W. Buddenhagen, CA; 7, A.R. Chase; 8, Cho, J. HI; 9, E. L. Civerolo; 10, M. A. Cubeta; 11, B. Dhanvantari, Canada; 12, E. Echandi, NC; 13, G. D. Franc, CO; 14, D. W.

Gabriel, FL; 15, R. D. Gitaitis, CA; 16, J. D. Janse, the Netherlands; 17, C. J. Kado, CA; 18, J. E. Leach, KS; 19, H. Maraite, France; 20, S. Nameth, Ohio; 21, W. T. Nishijima, HI; 22, S. Patil, HI; 23, D. J. Robeson, CA; 24, D. C. Sands; 25, M. N. Schroth, CA; 26, L. Sequeira, WI; 27, M. P. Starr, CA; 28, J. W. L. VanVuurde, The Netherlands; 29, W. H. Vong, China; 30, J. C. Watterson, CA; 31, P. H. Williams, WI; 32, R. S. Zeigler, Colombia.

Table 2.3. Antigen preparations made from *Xanthomonas oryzae* pv. *oryzae* strains to immunize mice for various fusions.

			No. of hybridomas		MAb
Fusion No.	Strain	Antigen type	Screened	Selected	Designation
175	PXO85	F[t]	190	None	-
176	PXO63	F	90	None	-
177	PXO35	F	191	None	-
178	PXO61	FPC[u]	333	None	-
187	PXO61	FPC/Am[v]	147	None	-
188	PXO61	FPC	262	None	-
189	PXO61	Son[w]	257	None	-
192	PXO61	Son/Am[x]	310	None	-
210	PXO61	Son/Am/cells[y]	310	1	Xoo-7
211	PXO35,PXO61, PXO63,PXO70, PXO79,PXO86, PXO105	F/L[z]			
226	T2	F/L	333	1	Xoo-9
232	NXO215	F/L	145	1	Xoo-11
239	NXO261	F/L	140	1	Xoo-10
240	NXO263	F/L	91	1	Xoo-8

[t] Formalin treated bacteria.

[u] French pressure cell disrupted bacterial extracts.

[v] French pressure cell extracts precipitated with saturated ammonium sulfate.

[w] Sonicated bacterial extracts.

[x] Sonicated bacterial extracts precipitated with saturated ammonium sulfate..

[y] Sonicated bacterial extracts precipitated with saturated ammonium sulfate and mixed with whole bacteria.

[z] Formalin treated and live bacteria.

Table 2.4. Serogroups of *Xanthomonas oryzae* pv. *oryzae* strains from various rice growing areas of the world.

	I[q]	IIa[r]	IIb[s]	III[t]	IV[u]	V[v]	VI[w]	VII[x]	VIII[y]	IX[z]	
X-1	+	+	+	+	+	+	+	+	+	+	299
Xco-1	+	+	+	+	+	-	+	+	+	+	297
Xco-2	+	-	-	+	-	-	+	-	+	-	242
Xco-5	-	-	-	+	+	-	-	-	-	-	27
Xoo-7	-	-	+	-	-	-	-	-	-	+	33
Xoo-8	-	-	+	-	-	+	-	-	-	+	35
Xoo-9	-	-	-	-	-	+	-	-	-	-	2
Xoo-10	-	-	-	-	-	-	-	-	+	+	15
Xoo-11	-	-	-	-	-	-	+	+	-	-	25
	192	6	30	15	14	2	23	2	12	3	

[q] 74 PXO strains (all races); 69 from India; 6 FXO strains; 22 from Australia, Bangaldesh, Burma, India, Indonesia, Japan, Taiwan, Thailand; 1 from Ceylon, CIAT 1186 from Columbia, 3 from Taiwan, IRN 395, XO3, XO24; 2 from Japan JXO T7133, T7174; 2 strains, A1779 and A1804; 14 Nepal strains (NXO101, 149, 160, 198, 200, 212, 216, 237, 256, 282, 331, 340, 356, 357).

[r] FXO strains 61, 63, NXO strains 161 ,174, 354 and Indian strain G 13.

[s] 14 Nepalese strains NXO 117, 142, 156, 157, 159, 180, 181, 194, 240, 259, 260, 263, 334, 355; 14 Indian strains GWB 11, 12, 13, 14, 16, 8203M, 8205M, 8209M, 8210M, 8212M, AP 19, AP 24, H100, H200; PXO 35 from the Philippines and CIAT 1185 from colombia.

[t] 15 Texas strains X1-5, X1-7, X7-5A, X7-2D, X7-3E, X11-1A, X11-5A, X11-1B, X11-4B, X11-5B, X11-6B, X11-2D, X13-3A, X13-5C, X13-2E.

[u] 10 Texas strains X1-6, X1-8, X1-10, X4-1B, X4-1C, X4-2C, X4-4C, X4-8C, X4-3D, X4-4D, Louisiana strains X8-1A, X8-1B, RU-8717, RU8718.

[v] Indian strains T1 and T2.

[w] PXO 5, 9, 63, 70, 79, 141, 146, 148, 152; NXO 239, 247; FXO 40, 65; Indian strains G1, 2, 3, 4, 5, 7, 8, 10, 8308M; Indonesian strain Ig-24.

[x] NXO 215, XO 20b.

[y] PXO 87; XO 16; NXO 151, 195, 196, 207, 228, 245, 261, 275; and 2 Indian strains (GWB 3, GWB 18).

[z] NXO 147, NXO 201; Indian strain 8327M.

Table 2.5. Characterization of monoclonal antibodies and the antigens they detected.

Antibody	Clone	Isotype	IF	IFC		Antigen	Other pathovars and X. *campestris*
				48h	72-96h		
Xco-1*	139-159*	IgMk*	Weak**	Weak	Weak	Protein moiety	None
Xco-2*	138-68*	IgG3k*	Bright**	Bright	Bright+halo	LPS**	None
Xco-5*	139-39*	IgG3k*	Intermediate*	ND		Heat and enzyme sensitive**	1
Xoo-7	211-G4	IgG3k	Bright	Bright	Bright+halo	LPS	None
Xoo8	240-40	IgG3k	Bright	Bright	Bright+halo	LPS	3 (8 strains)
Xoo-9*	226-42	$IgG2_ak$*	Negative	ND	ND	ND	None
Xoo10	239.11	IgG3k	Negative	ND	ND	Heat sensitive	None
Xoo11	232.D20	IgM	Negative	ND	ND	Heat resistant	5 (9 strains)

* Determined by Benedict et al. (1989)

** Determined in the present study and by Benedict et al. (1989)

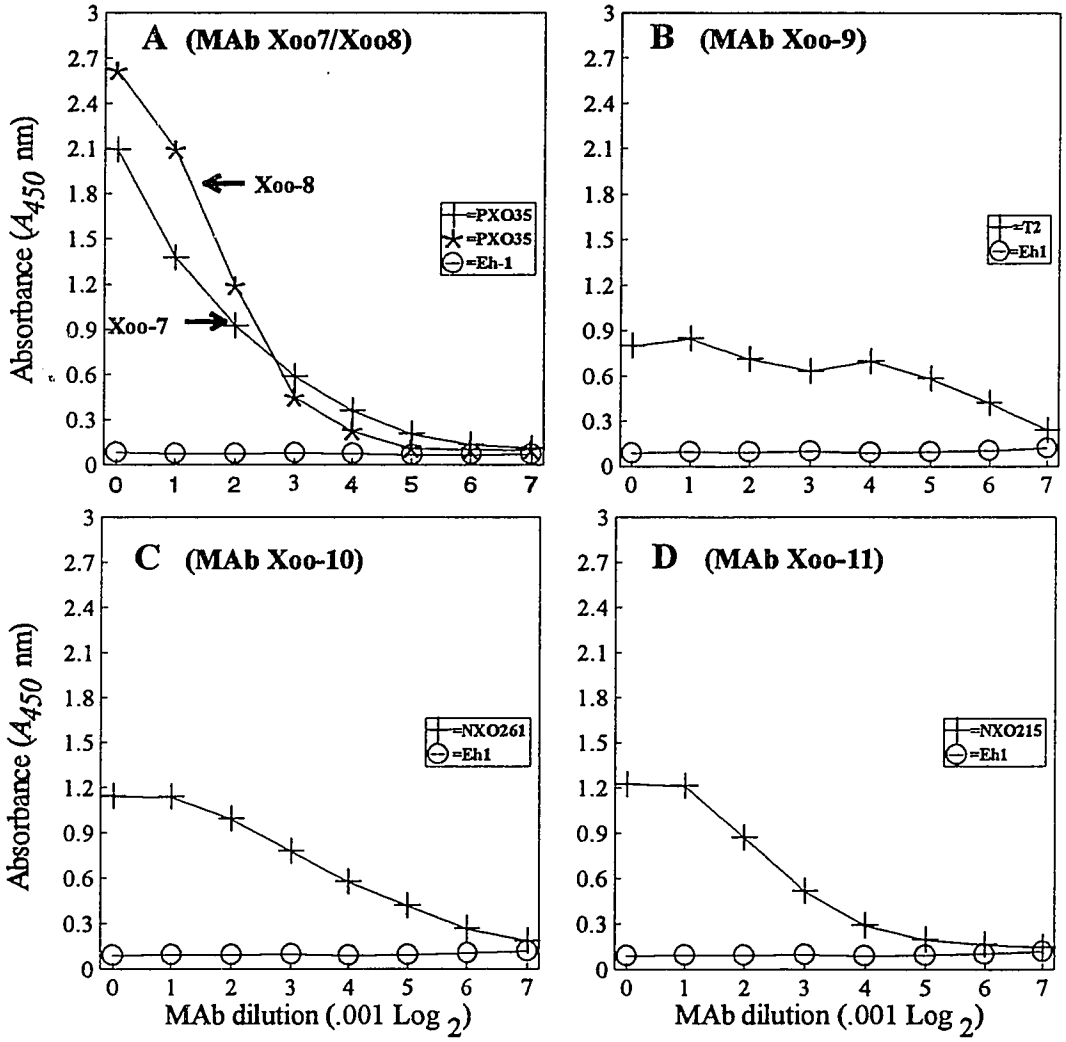

Fig. 2.1. Binding curves of monoclonal antibodies, Xoo-7 and Xoo-8, **A;** Xoo-9, **B;** Xoo-10, **C;** and Xoo-11, **D,** with *Xanthomonas oryzae* pv. *oryzae* strains PXO35, T1, NXO261, and NXO215, respectively. Absorbance readings for *Pantoea herbicola* strain (Eh-1) used as a negative control, were subtracted from absorbance values in above curves.

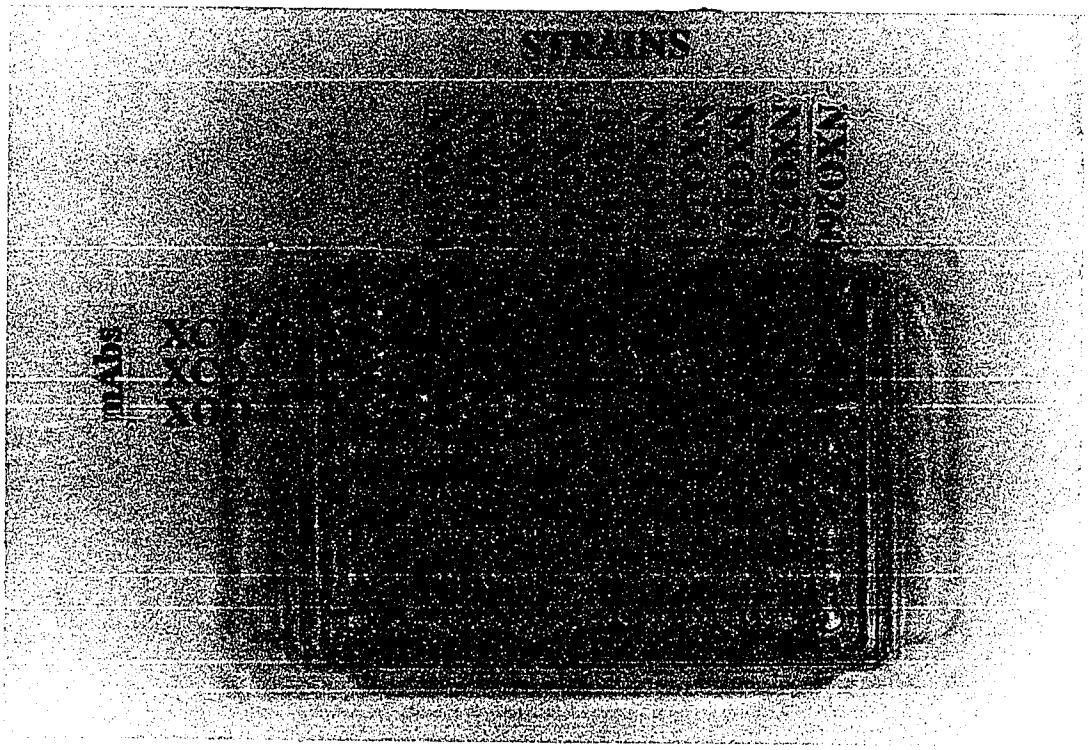

Fig 2.2. Reaction of monoclonal antibodies (MAbs) Xco-2 and Xoo-7 with *X. o. oryzae* strains from Nepal. The reaction pattern of 92% of the 299 strains tested were similar to that shown here (reacted with either MAb Xco-2 or Xoo-7) whereas the remaining strains were negative with both MAbs. None of the strains were positive with both MAbs. A pathovar specific MAb Xco-1 reacted with all the strains. The first two columns are negative controls.

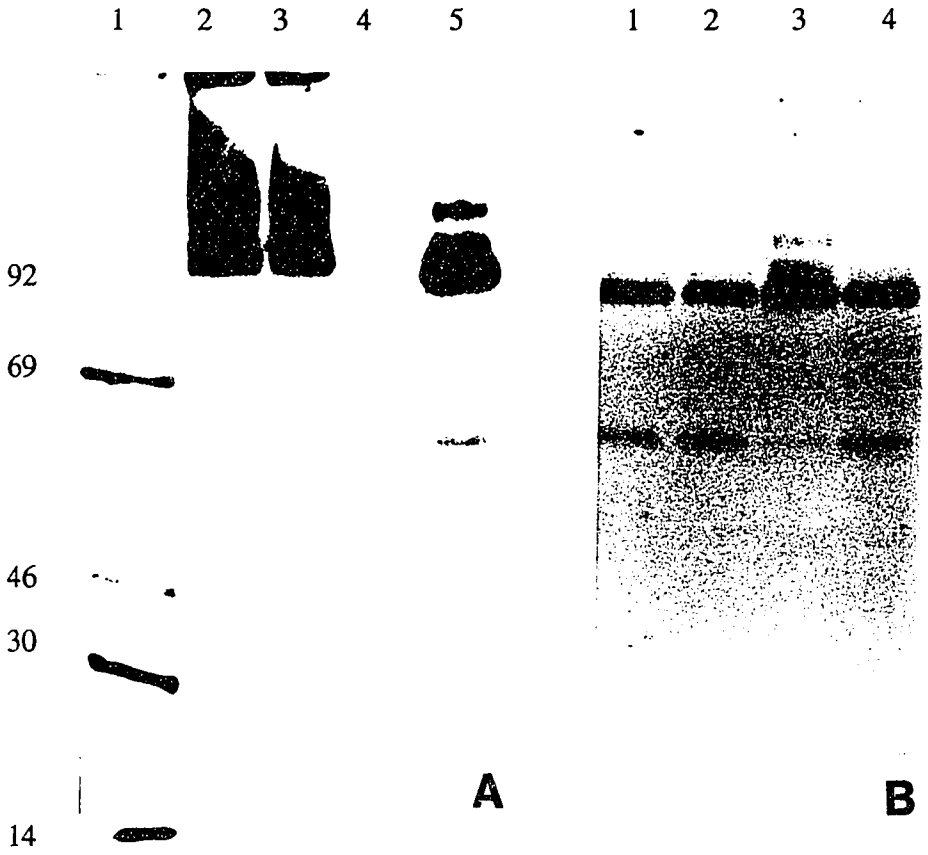

Fig. 2.3. Western blots (7.5% gel) of French pressure cell extracts of a *X. o. oryzae* strain X1-5 reacted with monoclonal antibody Xco-1. **A:** Lane 1, molecular weight markers (X 10^{-3}); lane 2, unheated, undiluted extract; lane 3, unheated extract diluted 1:2; lane 4, extract heated at 100 C for 3 min in 2X sample buffer; lane 5, unheated extract treated with proteinase K for 6 hr at 37 C; **B:** Lane 1, unheated extract treated with proteinase K; lane 2, unheated extract treated with pronase; lane 3, unheated extract first treated with pronase and then proteinase K; lane 4, unheated extract treated first treated with RNase for 20 min at 25 C and then with proteinase K.

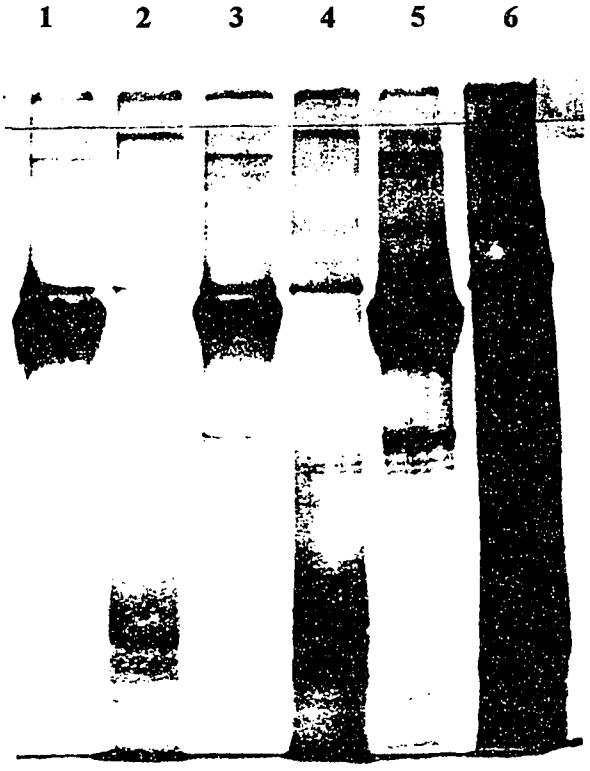

Fig. 2.4. Silver staining of proteins from French pressure cell extracts of a *X. o. oryzae* strain X1-5 in 7.5% gel after resolving by SDS-PAGE. Lanes 6, 4, and 2, unheated undiluted, diluted 1:2 and 1:4, respectively; lanes 5, 3, and 1, unheated extract treated with proteinase K for 6 hr at 37, diluted 1:2 and 1:4, respectively. The thick dark band as observed in Fig. 2.3A and B after enzyme treatment is visible in lanes 1, 3, 5.

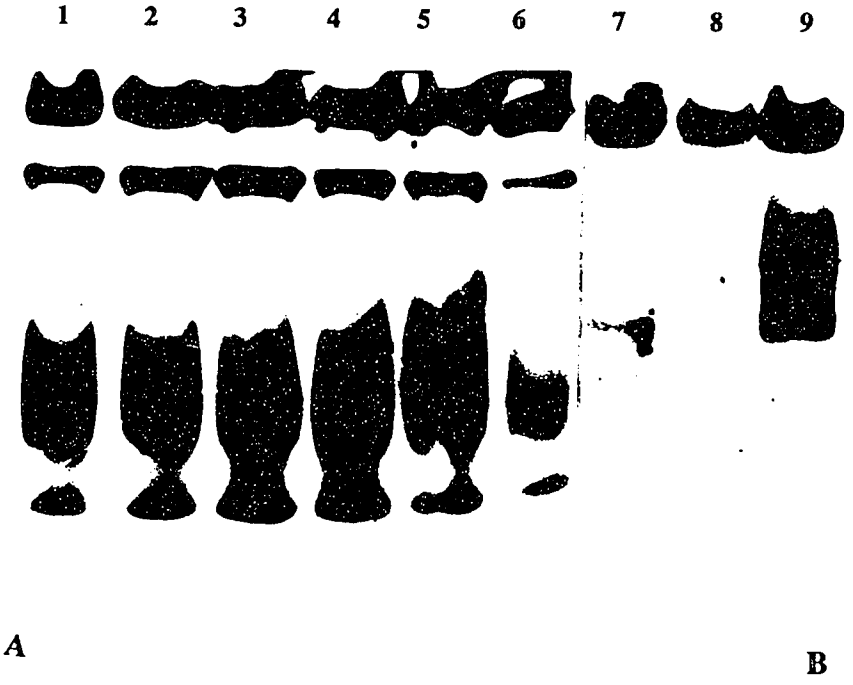

Fig. 2.5. Heat sensitivity of the antigen detected with monoclonal antibody Xco-1 in western blots of French pressure cell extracts of a *X. o. oryzae* strain. Lanes 1-6 are from 6% gel and lanes 7-9 from 7.5% gel. Lanes 1 and 9 unheated extracts. Lanes 2-7 extracts in phosphate buffered saline (PBS) without SDS, heated at 50, 60, 70, 80, 90 and 100 C for 3 min, respectively. Lane 8, extract heated at 50 C in PBS containing 0.1% SDS.

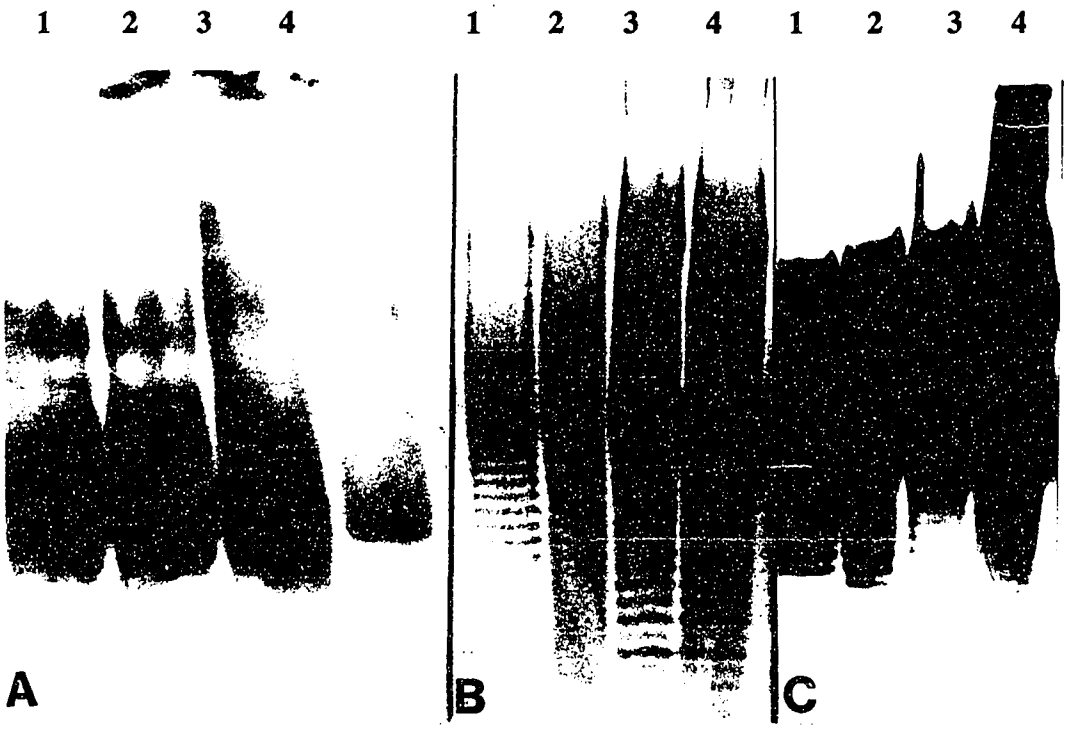

Fig. 2.6. Silver staining and western blotting of antigens detected with monoclonal antibody Xco-2. **A:** Western blot of French pressure cell extracts, lane 1, unheated extract treated with proteinase K for 6 hr at 37 C; lane 2, unheated extract treated with pronase E at 25 C for 15 min; lane 3, unheated extract treated with RNase at 25 C for 20 min; lane 4, diluted unheated extract. **B:** Silver stained SDS-PAGE gel (7.5%) of partially purified lipopolysaccharides (LPS) and **C:** corresponding western blot. Lanes B1 and C1, untreated LPS of *X. o. oryzae* strain X1-5; lanes B2 and C2, RNase/proteinase K treated LPS of X1-5; lanes B3 and C3, untreated LPS of *X. o. oryzae* strain PXO86; lanes B4 and C4, RNase/proteinase K treated LPS of PXO86.

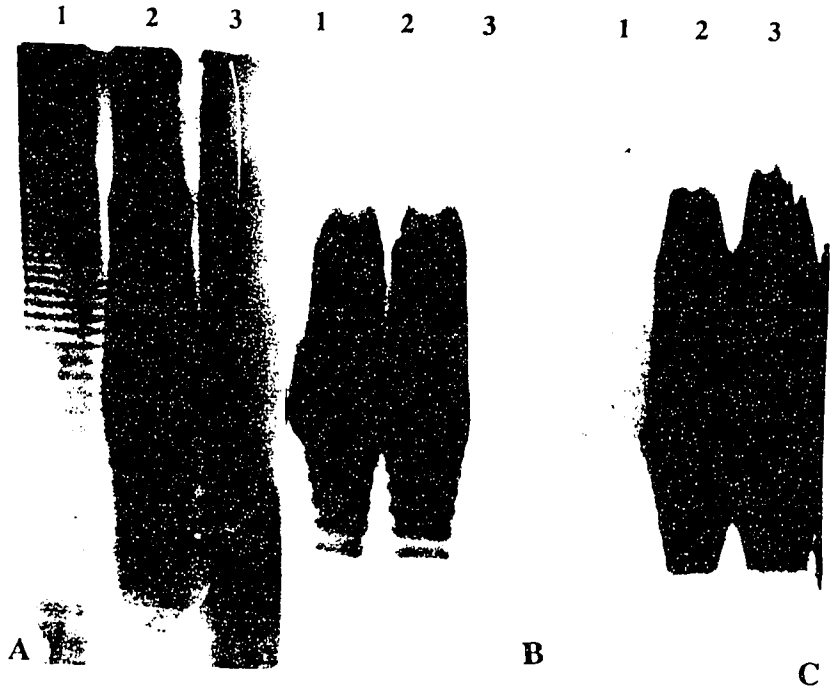

Fig. 2.7. Silver stained SDS-PAGE gel (7.5%), **A**, and corresponding western blots of partially purified lipopolysaccharide (LPS) of *X. o. oryzae* strain PXO35, detected with monoclonal antibodies Xoo-7, **B**; and Xoo-8, **C**. Lanes A1, B3 and C1 purified *Salmonella typhosa* LPS (Difco Laboratories, Detroit, MI). Lanes A2, B2 and C2, LPS treated with RNase (20 min at 25 C)/proteinase K (6 hr at 37 C) and centrifuged at 80,000 g; lanes 3A, 1B and 3C, untreated LPS.

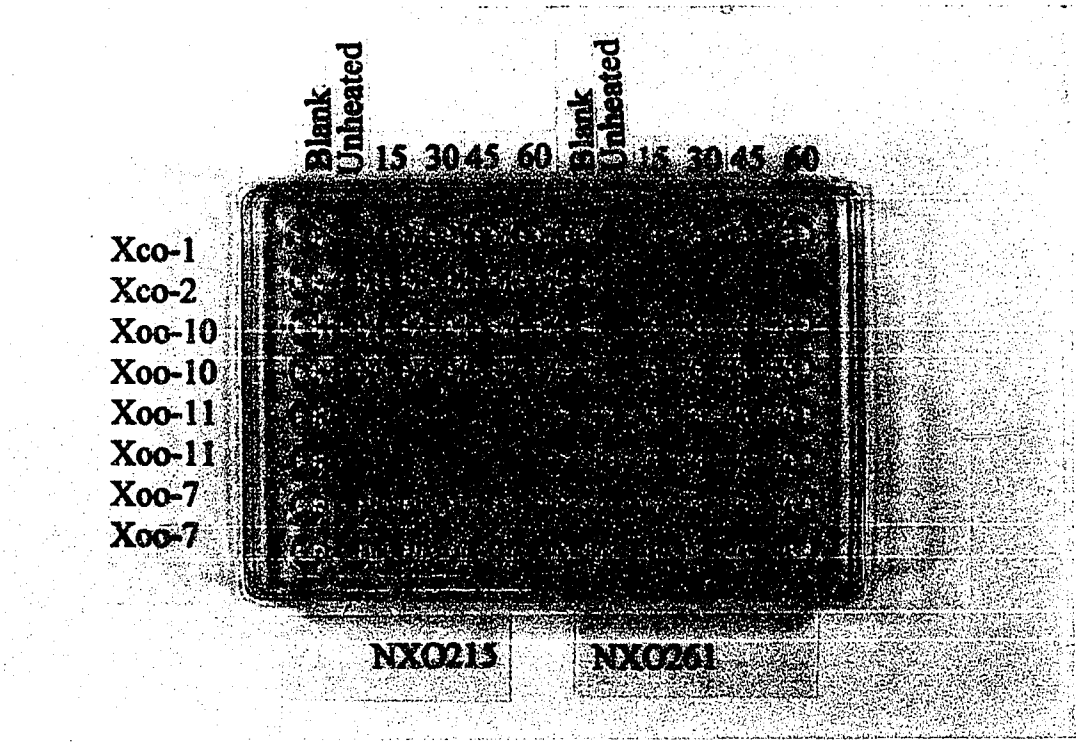

Fig. 2.8. ELISA reactions of monoclonal antibodies (MAbs) against unheated and heated extracts *X. o. oryzae* strains NXO215 and NXO261. The MAb Xoo-10 did not react with NXO261 when heated at 100 C. MAb Xoo-11 reacted with NXO215 even when heated up to 60 min at 100 C. MAb Xoo-7 was used as a negative control. MAb Xco-2 was used as a negative (NXO215) and positive (NXO261) control. MAb Xco-1 did not react with heated extract of either of the strains.

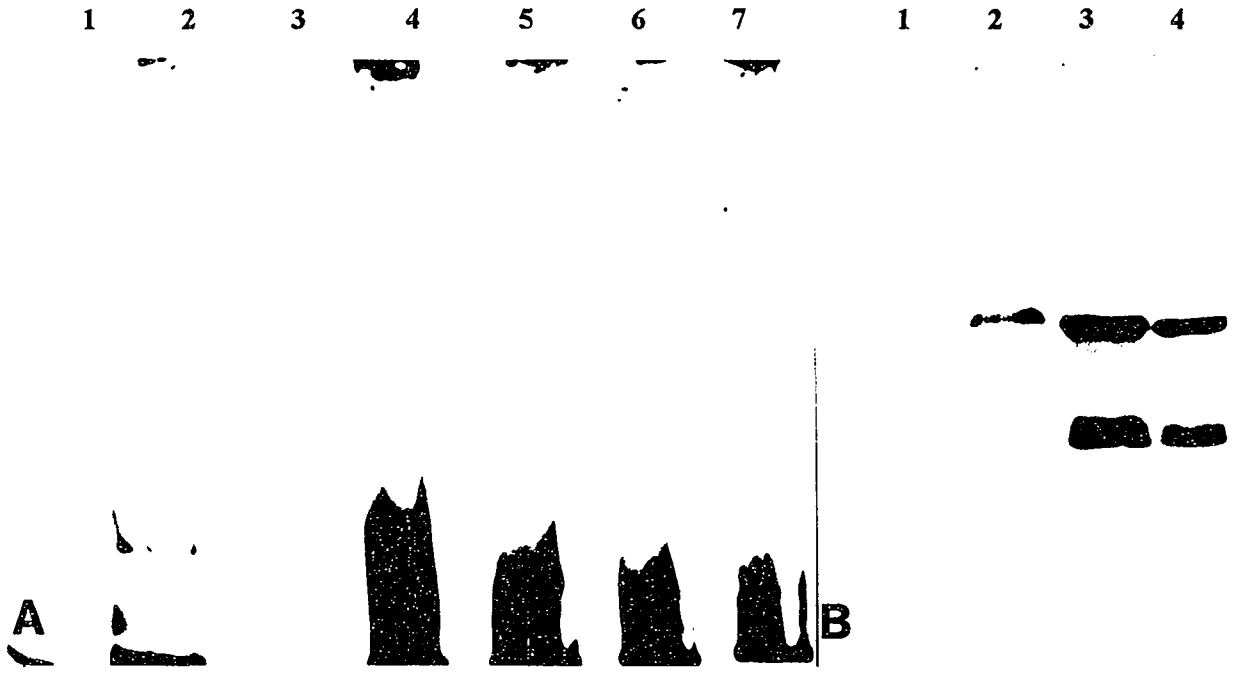

Fig. 2.9. Western blots of heated and sonicated extracts of *X. o. oryzae* strain NXO215 reacted with monoclonal antibody (MAb) Xoo-11. **A** (7.5 % gel), lane 1, unheated cells; lane 2, cells heated 3 min at 100 C in SDS-PAGE sample buffer (SB); lane 3, supernatant from the lane 2 preparations after centrifugation; lanes 4-7, cells heated at 100 C for 15, 30, 45 and 60 min, respectively. **B** (15% gel), sonicated extracts heated 3 min at 100 C in SB, diluted 1:4 and 1:2, lanes 1 and 2, respectively; 3 min heated extracts diluted 1:2 and 1:4, lanes 3-4, respectively.

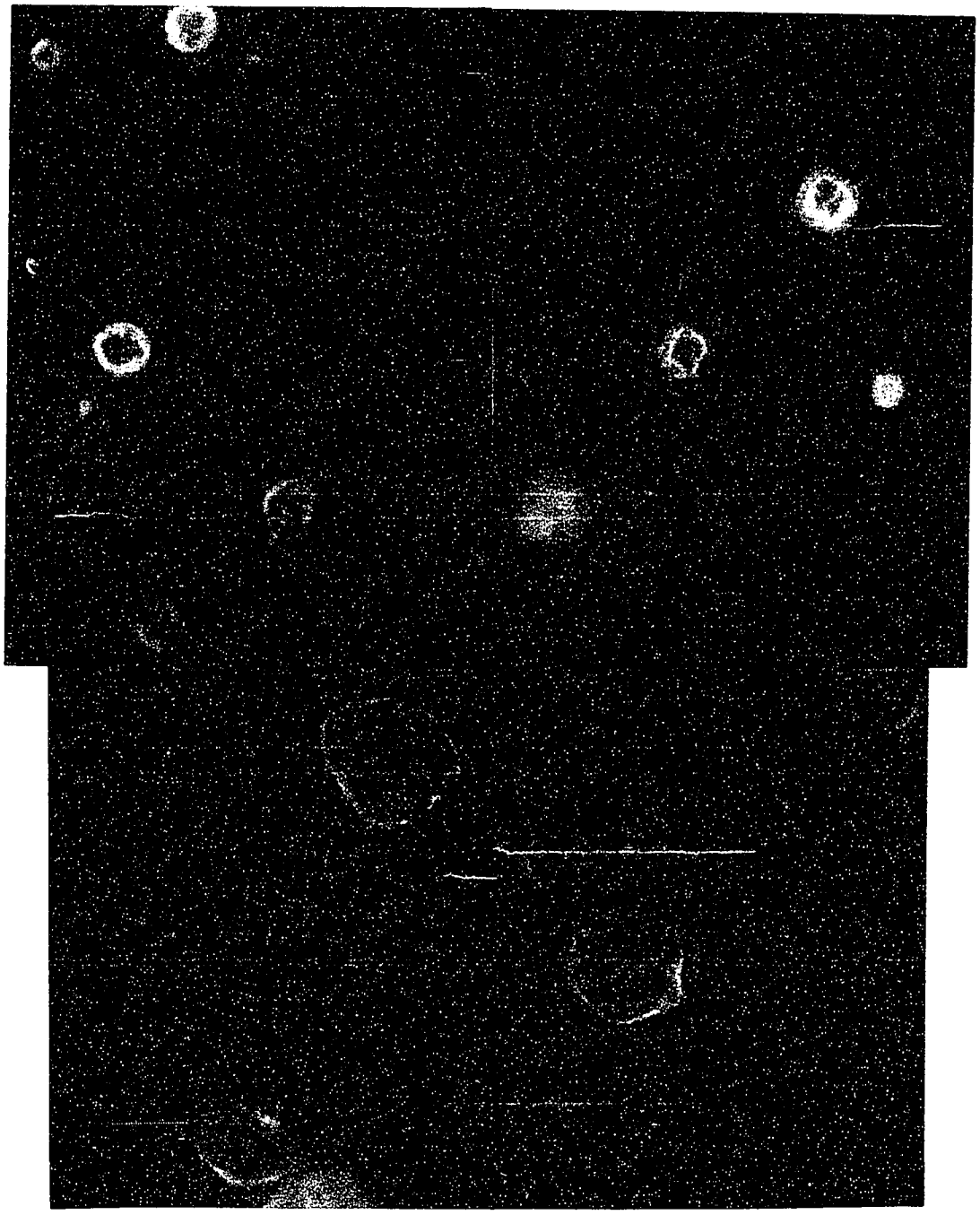

Fig. 2.10. Indirect immunofluorescence colony staining on 48 hr old cultures of *X. o. oryzae* strains PXO35 and PXO86. **A** and **B**, Monoclonal antibodies Xoo-7 and Xoo-8, respectively, reacting with PXO35; **C**, MAb Xco-2 reacting with PXO86.

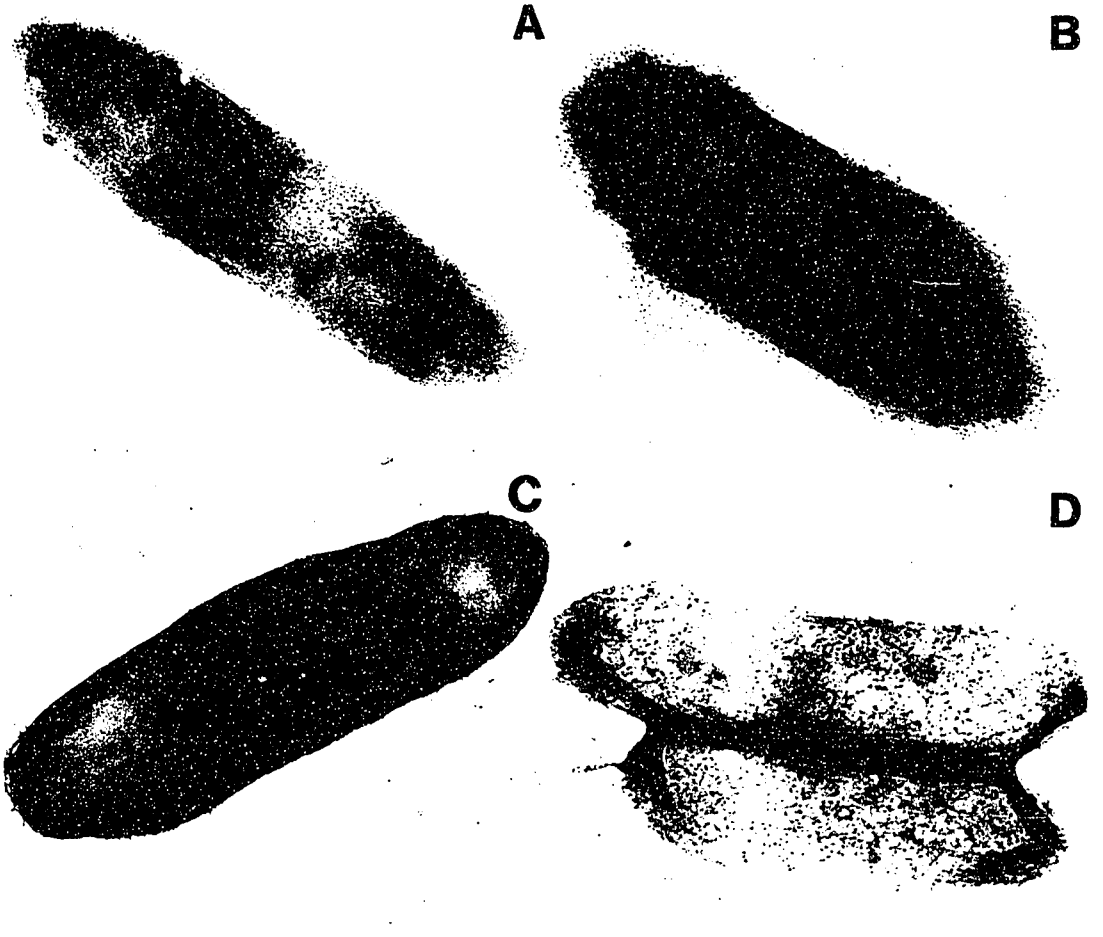

Fig. 2.11. Immunoelectron micrographs of *X. o. oryzae* strains reacted with monoclonal antibodies (MAbs). **A,** PXO35 + MAb Xoo-7; **B,** PXO35 + MAb Xoo-8; **C,** NXO261 + MAb Xoo-10; **D,** NXO215 + MAb Xoo-11.

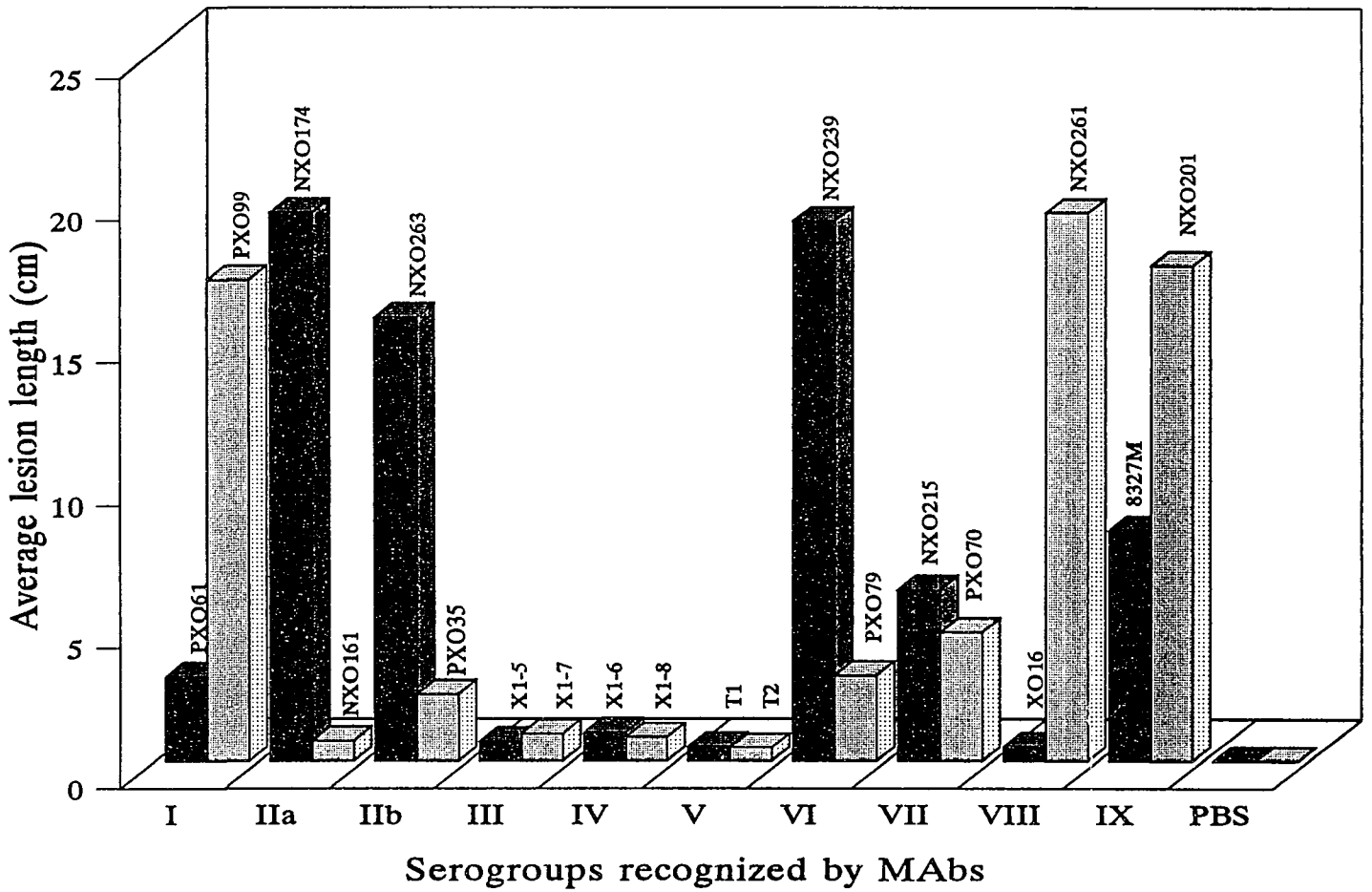

Fig. 2.12. Relationship of serogroups to virulence of *Xanthomonas oryzae* pv. *oryzae* on the IRRI rice cultivar IR 1545-39.

CHAPTER 3

Immunodetection of *Xanthomonas oryzae* pv. *oryzae* using a Mixture of Monoclonal Antibodies in a Immunofluorescence Colony Staining Technique.

ABSTRACT

An immunofluorescence colony staining technique (IFC) was developed using fluorescein isothyocyanate (FITC) conjugated monoclonal antibodies (MAbs), Xco-2 and Xoo-7, and a semiselective medium E, for detection of *Xanthomonas oryzae* pv. *oryzae* from rice seed extracts. IFC has a greater sensitivity than any other available detection technique for this pathogen because of an enrichment step in semiselective medium. Typical *X. o. oryzae* strains formed colonies on a semiselective medium E that were visible at 40X magnification in only 2 to 4 days as compared to the 5 to 8 days on other semiselective media. Colonies of typical *X. o. oryzae* were detected with IFC in extracts of artificially infested rice seed (1% infestation rate) containing 4.4 X 10^2 cfu/ml, even when plates were crowded with approximately 2300 contaminants. IFC also enabled detection of *X. o. oryzae* from extracts of 1000 rice seeds mixed with 25 mg fine powder of naturally infected rice leaves. Pathogenicity tests confirmed identity of stained colonies. Since both MAbs give bright fluorescence, a mixture of MAbs Xco-2 and Xoo-7, can be used with IFC to detect most of the typical strains *X. o. oryzae*. Enrichment on medium E followed by identification of *X. o. oryzae* by IFC with two pathovar-specific MAbs is a sensitive immunodetection method that can be used for epidemiological and seed transmission studies of *X. o. oryzae*.

INTRODUCTION

New outbreaks of bacterial blight (BB) of rice in previously disease free areas is attributed to the frequent seed exchange of improved rices among the rice growing countries (Leach et al., 1991). Although the causal bacterium, *Xanthomonas oryzae* pv. *oryzae* (Swings et al., 1990) is known to be seed borne (Mew et al., 1993), its transmission to the subsequent rice crop has been inconclusive and remains to be clearly demonstrated (Chakravarti and Rangarajan, 1967; Eamchit and Ou, 1979; Kauffman and Reddy, 1975; Goto, 1988; Mew et al., 1989; Ou, 1985). The common problem encountered in establishing the transmissibility of *X. o. oryzae* is its slow growth on isolation media. In contaminated culture, the pathogen is unable to compete with the fast-growing bacteria and its identification is often confused with other yellow-pigmented non-pathogenic xanthomonads that are often associated with rice seed (Benedict et al., 1989; Di et al., 1991; Gonzalez et al., 1991).

Efforts have been made to develop media for the selective growth and recovery of *X. o. oryzae* (Gonzalez et al., 1991; Yuan et al., 1990; Di et al., 1991). The XOS medium developed by Di et al. (1991) was considered most effective for direct isolation of *Xanthomonas* spp., including *X. o. oryzae,* and it suppressed some of the common saprophytes of rice seed (Di et al., 1991; Gnanamanickam et al., 1994). This medium was reported to aide in the detection of *X. o. oryzae,* from eight year old rice seed (Dikin et al., 1993). When comparing colony development of 12 strains of *X. o. oryzae*, however, typical blight-producing *X. o. oryzae* strains took more than 6 days to become visible and only atypical, weakly virulent strains formed colonies in three days. Colony development of a fast growing atypical strain was increased by increasing FeEDTA concentrations from 1 to 100 mg per liter, but growth of typical strains was reduced at higher levels of FeEDTA (Gnanamanickam et al., 1994). Growth of a typical strain PXO86, from the

Philippines, was not supported on a culture medium developed by Yuan et al. (1990). Thus, it is apparent that the media and assays for efficient and reliable detection and identification of typically slow growing strains of this pathogen still need improvement.

An immunofluorescence colony (IFC) staining technique has been used for direct isolation and identification of plant pathogenic bacteria (Van Vuurde, 1987; Mochizuki et al., 1992). In this technique fluorochrome conjugated, specific antibodies react with target bacterial colonies within an agar medium even when they are growing in the presence of interfering contaminants. The target colonies stained with antibodies are then identified by their bright fluorescence under UV light. The stained colonies also can be isolated for further identification and pathogenicity tests (Van Vuurde, 1987). Appropriate culture media and the availability of the specific antibodies against the target organism are important factors that determine the success of this technique.

Two monoclonal antibodies (MAbs) Xco-2 and Xoo-7, that gave bright fluorescence in indirect immunofluorescence (IF) and IFC and showed strong titers in ELISA, were available (Benedict et al., 1989; Rehman et al., 1992; Chapter 2). MAbs Xco-2 and Xoo-7 reacted with 85% and 12%, respectively, of 268 typical strains of *X. o. oryzae* when tested against 299 strains representing diverse geographical areas of the world. The strains that reacted with the MAb Xco-2 did not react with the MAb Xoo-7 and vice versa. Only 8 of the typical 268 strains failed to react with these MAbs (Chapter 2). Thus, a mixture of MAbs Xco-2 and Xoo-7 could be used to recognize at least 97% of the typical *X. o. oryzae* strains. The present studies were initiated to explore the possibility of detecting and identifying *X. o. oryzae* in contaminated seed extracts using IFC. Since IFC requires an enrichment step and since typical *X. o. oryzae* strains grew slowly in XOS medium, modifications were made to enhance the colony development of these strains for use in IFC.

MATERIALS AND METHODS

Bacterial strains and their cultural characteristics. The strains used in this study are given in Table 1. The *X. o. oryzae* strains from 10 serogroups (Chapter 2) were tested for pathogenicity on susceptible cultivars by the providers of the strains. The Nepalese and Indian strains were characterized at the University of Hawaii on differential rice cultivars (Rehman et al., 1993). Bacteriological, physiological, and pathogenicity tests were performed on predominant saprophytic and pathogenic bacteria isolated from rice seeds and leaf tissue (Table 1). The Biolog system (Biolog^{TM}, Inc. Hayword, CA), API Rapid NFT system (Plainview, NY) were used to characterize other representative strains. For use in the media development studies, bacterial strains of *X. o. oryzae* stored at -20 C were streaked on PSA (Tsuchiya et al., 1982), TZC (Kelman, 1954) or YDC agar media (Wilson et al., 1967) and incubated at 28 C for 24 to 72 hr. For serological analysis, strains were first transferred to YGA (Alvarez et al., 1978) and then processed for ELISA as described in Chapter 2.

Conjugation of monoclonal antibodies. The purification and conjugation of Ascitic fluids of MAbs Xco-2 and Xco-7 were fractioned and antibodies were conjugated using Goding's procedure (1986). Briefly, 4 ml of ascitic fluids of both MAbs were separately passed through a Sephadex G-200 column. The fractions containing immunoglobulin class IgG were pooled and concentrated under reduced pressure at 4 C. Protein concentrations were determined using a spectrophotometer, and fluorescein isothyocyanate-FITC, (Sigma, USA), was conjugated to the antibodies. The conjugated MAbs were separated from the unbound fluorochrome in a Sephadex G-75 column. The fluorochrome/protein (F/P) ratios were determined (Goding, 1986) and MAbs were stored at 4 C for use at a later time. Direct IF and IFC tests were performed to evaluate the

effectiveness of conjugation, using *X. o. oryzae* strain PXO86 to test MAb Xco-2 and strain PXO35 for MAb Xoo-7.

Detection of *X. o. oryzae* with an antibody mixture. Three methods, ELISA, IF and IFC, were used to compare limits of detection of a mixture of conjugated MAbs Xco-2-FITC and Xoo-7-FITC. For ELISA, the *X. o. oryzae* strains, PXO86 and PXO35, were first grown on YDC for 24 hr at 28 C, then bacterial suspensions were made in saline (0.85% sodium chloride). Suspensions were optically adjusted in a spectrophotometer to OD A_{600} = 0.1. Ten-fold dilutions were made, the 10^{-4} and 10^{-5} dilutions of both strains were mixed in 1:1 ratios and 0.1 ml from each of the mixed dilutions were plated on PSA. After 72 hr growth, single randomly picked colonies were suspended in 1 ml saline and processed for ELISA (Chapter 2) using 1:1000 dilutions of MAbs Xco-1, Xco-2, Xoo-7. In the mixture of Xco-2 and Xoo-7 the final dilution of each MAb also was 1:1000.

For direct IF, bacterial suspensions of PXO86 and PXO35 were coated individually or in a 1:1 mixture on glass slides and IF was performed as described in Chapter 2 except that the directly conjugated MAbs Xco-2-FITC and Xoo-7-FITC were used rather than rabbit anti-mouse-FITC secondary antibody.

Procedures for IFC (Van Vuurde, 1990) were used with modifications. Pure and mixed cultures (1:1) of PXO86 and PXO35 were grown on PSA medium for 72 to 96 hr. The 1:50 or 1:100 dilutions of MAbs Xco-2-FITC and Xoo-7-FITC and 1:25 or 1:50 dilution of a 1:1 mixture of two MAbs were used to stain dried agar pieces (approximately 1 cm^2) containing pure or mixed cultures of both strains. The procedure was performed in a 24 well sterile tissue culture plate covered with aluminum foil and the plate was kept at room temperature on a rotary shaker (200 rpm). An agar piece containing a mixed culture was first stained with MAb Xoo-7-FITC for 12 hr and observed for the stained colonies under UV light, then treated for another 12 hr with MAb Xco-2-FITC to stain the remaining colonies.

Evaluation of growth media. Media XOS (Di et al., 1991), modified XOS (Gnanamanickam et al., 1994), and PSA (Tsuchiya et al., 1982) were evaluated using nine typical (PXO86, PXO35, PXO70, NXO215, NXO239, NXO261, G13, G14A, A8327) and three atypical (X1-5, X1-8, T1) *X. o. oryzae* strains. Ten-fold dilutions of bacterial suspensions of these strains were made as described above and 10 µl (approximately 1 cm^2) were spot-plated onto each of the media from 10^{-4} to 10^{-5} dilutions in (10 replications) using an EPD electronic pipette (Rainin Instruments Co., Woburn, MA). Colony forming units (cfu) were counted under dark field illumination at 40X magnification, and data on the rate of colony appearance were recorded for 10 days. Plating efficiencies of XOS and modified XOS media were determined by comparison with the efficiency of PSA medium.

Composition of media used to analyze some of the individual components of XOS medium are given in Table 3.2. Initially, 5 strains were used to observe colony development. Medium-E (without K_2HPO_4) was further compared with medium E1 (only 0.2 g/L K_2HPO_4) and XOS medium to determine the effect of K_2HPO_4 on growth, rate of colony appearance, and colony morphology of these strains. Finally, all of the strains were tested on media E, E1, and XOS. Media E and E1 were also modified with 0.001% tetrazolium chloride (TZC) and two predominant contaminants of rice seed and a *X. o. oryzae* strain were tested to ascertain if TZC aided in differentiation from *X o. oryzae*. In all experiments, 10 µl spots were plated in 5 or 10 replications, the rate of colony appearance was recorded and final colony counts were determined at 7 days. Some experiments were repeated once, others twice.

Detection of *X. o. oryzae* from the seed extracts mixed with a pure culture of PXO86. Rice seeds were harvested from rice cultivar IR 1545-39 grown in a disease free area of a green house at the University of Hawaii. To obtain the extract, 25 g (approximately 1000) seeds were placed in a 500 ml plastic beaker containing 50 ml saline, crushed with a pestle for 3 min and incubated for 2 hr at 4 C on a rotary shaker adjusted

to 200 rpm. The extract was decanted from the seed and centrifuged at 17,000 g for 30 min. The pellet was re-suspended in 1 ml saline and 10-fold dilutions were made from these cells as well as from a 24 hr culture of *X. o. oryzae* strain, PXO86. A constant number of *X. o. oryzae* from the 10^{-4} dilution was mixed with 10-fold dilutions of seed extract to obtain samples (1 to 4). An aliquot of 0.1 ml from each sample was plated in four replications. Two methods of plating, standard spread plate and pour plate (for IFC) were used. For pour plating, medium E with 1.2% agar, was cooled to approximately 37 C. The bacterial suspensions or seed extracts containing *X. o. oryzae* were placed in the middle of 90 mm sterile petriplates, and approximately 12 ml of the medium added per plate. Plates were immediately swirled to spread the suspensions prior to solidification of agar. Plates were incubated at 28 C and the number of cfu were recorded from 1 to 7 days. The expected numbers of cfu/ml for contaminants and *X. o. oryzae* were determined by plating seed extract or the suspension of PXO86 separately on medium E (four replications). The IFC was used when *X. o. oryzae* colonies were not visually detectable due to the presence of too many contaminants. For IFC, when pinhead colonies of *X. o. oryzae* became visible in E1 medium, agar pieces (approximately 1 $cm^{2)}$ containing target colonies were stained with MAb Xco-2-FITC. The suspected colonies observed by both methods were then streaked on YDC to be compared with pure cultures of PXO86 and to tested with direct IF. The experiment was repeated.

Detection from artificially infested seed. Rice seeds from the same batch as above were autoclaved for 20 min under 15 psi at 121 C, plated on YDC to check the contaminants and incubated at 28 C for 4 days. Three hundred sterilized rice seeds were then soaked in 5 ml bacterial suspension of a *X. o. oryzae* strain PXO86 that was grown for 24 hr on PSA and adjusted to OD A_{600} = 0.1. Seeds were dried in a laminar flow hood overnight, and approximately 30 seeds were plated on YDC to test the infestation efficiency. They were stored in a desiccator at 4 C under sterile conditions. Five samples

(100 seed per sample) containing 0, 1, 5, 10, or 100 infested seeds were obtained by mixing artificially infested seed with non-sterilized seed. Seeds were placed in 200 ml plastic beakers containing 5 ml sterile saline and processed as described before to get seed extract. Ten-fold dilutions were made from seed extracts and 0.1 ml extract from all the dilutions of each sample were pour plated using medium E. Visual counts of cfu were recorded from 10^{-3} dilutions for 7 days and the average of five replications was used to calculate cfu/ml in undiluted extracts. IFC was performed on the agar pieces taken from all the dilutions of each sample. Experiment was repeated with five samples of 1% infested seed and one sample from each of 0, 5, and 100% infested seed. The artificially infested seeds used in this experiment to prepare all the samples were the same as in the first experiment, except that the seeds were kept at 4 C in a desiccator for 10 months.

Detection of *X. o. oryzae* from diseased leaf tissue mixed with seeds harvested from naturally infected rice plants. Symptomatic leaves and rice seed of naturally infected cultivar IR-20 were by S. S. Gnanamanickam from India. Leaves were observed for bacterial streaming under a stereomicroscope and the ooze from leaves was tested with MAbs Xco-2-FITC and Xoo-7-FITC in direct IF. Fifty rice seeds were weighed individually to determine the average weight per seed. Finely ground leaf powder of symptomatic leaves equivalent to the weight of one seed (0.025 g) was mixed with 25 g of seed (approximately 1000 seeds) and placed in 50 ml saline. Leaf powder alone (0.025 g) in 50 ml saline was used to determine the cfu/ml *X. o. oryzae* in leaf powder and 25 g seed alone in 50 ml saline were used to determine cfu/ml of contaminants in seed. Extracts of contaminated seed were obtained as described above except that no centrifugation was performed. Ten-fold dilution were made and 0.1 ml from each dilutions pour plated in medium E (four replication). A similar experiment was conducted to compare detection on two media (E and XOS). Expected numbers of cfu/ml were determined from the controls, and IFC was performed. Colonies of presumptive *X. o. oryzae* and other contaminants

were streaked on YDC for comparisons of colony morphology, pathogenicity and reaction in direct IF. Pathogenicity tests were performed by scissor-clipping method (Kauffman et al., 1973) using rice cultivar IR-20. A *X. o. oryzae* strain NXO239 and sterile saline served as positive and negative controls, respectively.

RESULTS

Detection of *X. o. oryzae* with an antibody mixture. ELISA reactions for 44 randomly selected colonies of mixed cultures of PXO86 and PXO35 are depicted in Figure 3.1. MAbs Xco-2 and Xoo-7, when tested separately, reacted only with the homologous strains and showed reciprocal reactions, whereas a 1:1 mixture of these MAbs reacted with all the colonies and showed the specificity obtainable with a pathovar specific MAb Xco-1 (Benedict et al., 1989). Strains were similarly differentiated with direct IF. Bright fluorescence of the homologous strains was observed when the two MAbs were tested separately by IF and IFC whereas both strains fluoresced when MAbs were used as a 1:1 mixture (data not shown).

In IFC experiments, 1:1 mixtures of PXO86 and PXO35 resulted in colonies of two different sizes at 96 hr of incubation in PSA agar (Fig 3.2A). When reacted with MAb Xoo-7-FITC, only small colonies of the homologous strain-PXO35 gave bright fluorescence whereas the two large colonies of the heterologous strain, PXO86, did not react (Fig. 3.2B). The latter colonies also gave bright fluorescence when the agar piece was stained for another 12 hr with MAb XCO-2-FITC, homologous to the strain PXO86 (Fig 3.3). When the antibody mixture was applied every colony in the mixed cultures gave bright fluorescence (Fig. 3.4).

Evaluation of growth media. Plating efficiency of 9 typical and 3 atypical *X. o. oryzae* strains on XOS and modified XOS medium are shown in Table 3.3. The plating efficiency of these strains on XOS (with 1 mg FeEDTA/L) medium ranged from 66-96%

of the recovery on PSA (Table 3.3). Efficiencies for atypical strains were not appreciably different from typical *X. o. oryzae* strains. Substantial differences, however, were observed in the rates of colony appearance. While atypical strains took only 3 days to become visible at 40X magnification, the typical strains formed colonies in 5 to 8 days with most of the strains appearing on day 7 (Table 3.3). A significant delay in the rate of colony appearance of the typical strains of *X. o. oryzae* occurred on all the media that contained 2.0 g/L K_2HPO_4 (media D, F, G, XOS, XOSM1, and XOSM2) as compared to media (A, B, C and E) that lacked phosphate (Tables 3.3 and 3.4). While colonies of typical strains appeared in 5 to 8 days on phosphate containing media, they took only 2 to 4 days to become visible on the media without phosphate. The maximum delay in the rate of colony appearance was observed on medium D that contained phosphate but lacked FeEDTA and $Ca(NO_3)_2.4H_2O$. Among the media that lacked phosphate but contained methylviolet-2B and antibiotics, earliest colony formation for typical *X. o. oryzae* was observed on the medium E (Table 3.4). The colony counts for all the strains, except G14A, were lowest on medium D (Table 3.4). Peptone at 10 g/L in XOSM2 medium retarded the growth of the typical strains, PXO35, NXO215 and G14A, but growth was unchanged on XOSM1 medium that contained 5 g/L of peptone.

The delayed appearance of typical *X. o. oryzae* strains observed on the media containing phosphate was further verified by comparing medium E and XOS with an additional medium E1 that contained K_2HPO_4 at 10% of the original concentration (Table 3.5). The colony counts and rates of colony appearance of all five strains were essentially the same on medium E and XOS as observed before. Medium E1 also accelerated the rate of colony appearance of typical strains *X. o. oryzae* (Table 3.5). In additional testing of media E and E1, all 12 *X. o. oryzae* strains formed colonies in 2 to 4 days but the final colony counts at day 8 were not significantly different from XOS (data not shown). The colonies of *X. o. oryzae* were mucoid, translucent, glistening, raised and round with

smooth margins on medium E after 4 to 5 days. Colonies of *X. o. oryzae* on medium E1 were similar in morphology to the colonies on medium E except that they were yellow on medium E1 (Fig. 3.5).

A common rice seed contaminant, *Pantoea (Erwinia) herbicola*, associated with rice seed did not grow on medium E, E1 or XOS (Table 3.6). In contrast, a commonly encountered fluorescent pseudomonad, identified as *Pseudomonas putida* with bacteriological tests and Biolog^{TM}, grew rapidly on all three media (Table 3.6). Pure cultures of *Pseudomonas putida* were inhibitory to *X. o. oryzae* strains and produced a large zone of inhibition when co-inoculated in an agar plate with PXO86 (Fig. 3.6). Incorporation of TZC into these media did not enhance differentiation of colonies and it masked the translucence or yellow color of *X. o. oryzae* on media E and E1. Addition of TZC in the medium E and E1 did not affect the final number of cfu/ml recorded on these media (Table 3.6). Medium E (without TZC) was selected for IFC because the colony diameters of *Pseudomonas putida* were smaller on the medium E as compared to medium E1 (Fig. 3.7) and the addition of 0.2 g/L phosphate (medium E1) did not enhance growth rate or final recovery of the twelve *X. o. oryzae* strains (Table 3.7).

Detection of *X. o. oryzae* from the seed extracts mixed with a pure culture of PXO86. Several types of contaminants were encountered in seed extracts. A dark yellow and a mucoid white contaminant were predominant from the samples. The dark yellow contaminant was identified as *Pseudomonas paucimobilis* by Biolog^{TM} system (0.625 similarity) and Rapid NFT strips and a white contaminant was identified as *Xanthomonas* sp. by Biolog^{TM} and other bacteriological tests (oxidative metabolism, esculin hydrolysis, oxidase negative, starch hydrolysis, negative for NO_3 reduction and positive reaction with genus specific MAbs X1 and X11). Colonies of commonly encountered contaminants developed in three days. Pinhead colonies of *X. o. oryzae* also became visible at 40X magnification on the third day. The differential size of *X. o. oryzae* and contaminants

enabled easy recognition of presumptive *X. o. oryzae* colonies under dark field illumination (Fig. 3.8). On the spread plate medium, the colonies of *X. o. oryzae* were translucent and very small compared to the contaminants, and they were recognizable on the third day at 40X magnification. The agar squares containing target colonies, cut from all the dilutions of each sample, gave bright fluorescence in IFC whereas contaminants remained unstained (Fig 3.9). The expected and detected number of cfu/ml for both contaminants and *X. o. oryzae* are presented in Table 3.8. Smaller colonies of *X. o. oryzae* were visually recognizable in samples 3 and 4 when the number of contaminants was low, but it was difficult to visually differentiate between contaminants and *X. o. oryzae* in samples 1 and 2 because of the high number of contaminants. Nonetheless, the colonies of *X. o. oryzae* from the latter samples were detected with IFC even when they were growing at ratios of 1:108 (Table 3.8) and 1:120 (Table 3.9) of *X. o. oryzae* to contaminants. The suspected *X. o. oryzae* colonies randomly picked from spread and pour plate medium E and grown on YDC were identical in colony morphology with the pure cultures of PXO86, and also gave bright fluorescence in direct IF. In a comparison between the two types of plating methods (pour plate and spread plate), a higher number of cfu/ml were obtained with the pour plate method in both experiments (Tables 3.8 and 3.9).

Detection from artificially infested seed. Sterile rice seeds that were artificially infested with *X. o. oryzae* strain PXO86 showed 100% infestation on YDC agar medium. The inoculated strain was recovered at 5.1 X 10^3 cfu/seed. Extracts from samples 1 to 4 yielded the same two predominant contaminants that were observed in previous experiments. The colony numbers from undiluted extracts ranged between 2.6 X 10^5 to 3.3 X 10^5 cfu/ml (Table 3.10). Recovery of *X. o. oryzae* from the same samples ranged from 8 X 10^3 to 5.1 X 10^5 cfu/ml. Although colonies of *X. o. oryzae* were visually detectable from 10^{-3} dilutions of samples 2 to 5, they were detectable only with IFC from

the lower dilutions of each sample where visual detection of *X. o. oryzae* colonies was difficult.

Colony counts by visual observations on medium E were not possible in a second experiment because the population of *X. o. oryzae* was approximately 91% lower than the experiment 1 (4.4 X 10^2 cfu/seed). Nonetheless, with qualitative use of IFC, colonies of *X. o. oryzae* were detected from all infested samples (Table 3.11). Eighty seven *X. o. oryzae* colonies showed bright fluorescence in a plate contaminated with approximately 2300 contaminant colonies (Fig. 3.10).

Detection of *X. o. oryzae* from diseased leaf tissue mixed with seeds harvested from naturally infected rice plants. In initial experiments, *X. o. oryzae* was not detected from extracts of two samples of approximately 1000 naturally infected rice seed whereas abundant bacterial ooze was observed in infected leaves. The ooze from infected leaves gave fluorescence only with MAb Xco-2-FITC, when tested with direct IF; therefore, this MAb was used in IFC to detect *X. o. oryzae* from seed extract contaminated with leaf tissue. Several types of contaminants also were isolated at the lower dilutions from the seed extract of rice cultivar IR-20. One of the two most commonly isolated contaminants was identified by the Biolog™ and Rapid NFT system as *Pseudomonas paucimobilis* (0.667 similarity). The other was identified by Biolog™ as *Xanthomonas campestris* pv. *strelitizia* (0.958 similarity). This xanthomonad had other characteristics of *Xanthomonas campestris* (oxidative metabolism, esculin hydrolysis, oxidase weak, starch hydrolysis, negative for NO_3 reduction and positive reactions with genus specific MAbs X1 and X11), but they were not pathogenic on *Strelitizia reginae.* Thus, the closest identification was *X. campestris.* Larger colonies of both contaminants formed in 2 to 3 days. The *X. o. oryzae* isolated from leaf tissue, however, appeared in 3 to 4 days as pinhead colonies at 40 X magnification. Although colonies of *X. campestris* were similar in morphology to *X. o. oryzae*, the differential rate of colony appearance and colony size aided visual

differentiation between the two organisms on medium E. Extracts obtained from the leaf tissue alone had high population densities of *X. o. oryzae*, with only a few contaminants at lower dilutions, and they yielded pure cultures at high dilutions (7.2×10^2 cfu/ml in 25 mg of leaf tissue) (Tables 3.12). Likewise, 1.3×10^6 cfu/ml of contaminants were detected from undiluted extract of seed alone. In the extract obtained from seed infested with 25 mg leaf tissue, the colonies of *X. o. oryzae* were not visually detectable at lower dilutions because plates were crowded with contaminants but they were readily detected with IFC. Results similar to the first experiment also were obtained in a second experiment with medium E (Table 3.13). No *X. o. oryzae*, however, was detected up to seven days in XOS medium although contaminants appeared at the same rate as they appeared on medium E (Table 3.13).

The presumptive *X. o. oryzae* colonies were pale yellow, mucoid, raised and round, with smooth margins on YDC and looked identical to the colonies from pure cultures of *X. o. oryzae* isolated from leaf tissue. They all gave bright fluorescence in direct IF and produced typical leaf blight symptoms on rice plants (Fig 3.11). Plants inoculated with NXO239 also produced typical bacterial blight symptoms, whereas saline control, *P. paucimobilis* and *X. campestris* were negative in both tests (Table 3.14).

DISCUSSION

The semi-selective medium XOS was developed for direct isolation of *Xanthomonas* spp., including *X. o. oryzae* from rice seed (Di et al., 1991). As observed previously (Gnanamanickam, et al., 1994) and in the present studies, this medium was suitable only for fast growing atypical strains of *X. o. oryzae* and other xanthomonds associated with rice seed (Jones et al., 1989; Gonzalez et al., 1991; Gnanamanickam et al., 1993). While atypical strains took 3 days to form colonies on XOS medium, the colonies of typical strains mostly formed in 7 days. The delay in the rate of colony appearance of

typical strains on XOS medium was attributed to the amount of phosphate in XOS medium. Yuan (1990) studied the effect of different phosphate salts and combinations on growth of *X. o. oryzae* and observed a delay in colony appearance with higher concentrations of phosphate salts. He thought that the affect on growth may have been partially due to the different pH (6.0-7.2) that these salts gave to the media.

In the present studies, the rate of colony appearance was substantially improved when phosphate was completely eliminated from XOS medium (medium E) or reduced to 0.2 g/L (medium E1) because colonies of typical strains then formed in 2 to 4 days. Moreover, the complete elimination of phosphate reduced the colony diameters of pseudomonads that are often associated with rice seed, whereas another common saprophyte from the rice seed, *Pantoea herbicola,* did not grow on this medium at all. A gene that produces a high-affinity phosphate-binding protein is induced in an enteric bacterium, *Escherichia coli,* when environmental levels of phosphate are very low (Makino et al., 1988; Warner, et al., 1987). The protein enables *E. coli* to grow in phosphate limiting conditions. The pathogen *X. o. oryzae*, like *E. coli*, exists in nature in conditions of phosphate starvation (Ou, 1985). A homolog of a *E. coli* gene producing phosphate binding protein also was found in *X. o. oryzae* and partially characterized recently (Hopkins et al., 1995). The ability of *X. o. oryzae* to grow and form colonies at a faster rate under low phosphate conditions may be related to this gene. Such manipulation of inorganic phosphate has, therefore, enhanced the competitiveness of *X. o. oryzae* and improved its recovery over any of the previously reported media (Gonzalez, 1991; Di, et al., 1991; Gnanamanickam et al., 1994; Yuan, et al., 1990) for the earlier recovery of typical *X. o. oryzae.*

Medium E in pour plates showed greater recovery than spread plates probably because bacteria utilized more surface area and spread in three dimensions. The differential rate of appearance and size of *X. o. oryzae* colonies observed in pour plates aided in the

visual detection of presumptive *X. o. oryzae* colonies when contaminant populations were low in number. However, due to its similarity in colony morphology to non-pathogenic xanthomonads, visual discrimination between these two types of organisms was difficult when plates were crowded with contaminants. Using IFC however, *X. o. oryzae* was readily detected even when present in a 120:1 contaminant to *X. o. oryzae* ratios.

Immunofluorescent colony staining technique is 1000-fold more sensitive than ELISA and detected as low as 4.4×10^2 cfu/ml of *X. o. oryzae* from rice seed extract. This method also enabled detection of 87 colonies of *X. o. oryzae* growing among thousands of contaminants in a pour plate. Besides higher sensitivity, the colonies of target bacteria were viable after staining (Van Vuurde, 1987) and were isolated from such pales to confirm identity. The direct conjugation of MAbs Xco-2 and Xoo-7 to FITC increased efficiency of IFC and eliminated intermediate steps (Van Vuurde, 1987). Staining of smaller agar pieces, instead of a whole plate, required less antibody and hastened the drying and staining procedure. It was necessary to stain the whole agar plate when target colonies were not detected from small agar pieces, however.

When rice seed is collected from rice fields, there is always a possibility that such seed may contain mixed populations of different strains. These strains may contain populations of *X. o. oryzae* that are serologically distinct (Benedict et al., 1989; Rehman et al., 1992, Chapter 2). It is therefore, important to use antibodies that can react with all the strains in a mixed population. A pathovar specific antibody, Xco-1 (Benedict et al., 1989) reacted with nearly all *X. o. oryzae* strains when 299 strains representing diverse rice growing areas of the world. This MAb, however, was less useful for IF than MAbs Xco-2 and Xoo-7 because the latter gave bright fluorescence in indirect IF and IFC (Chapter 2). Since MAbs Xco-2 and Xoo-7 reacted with 85% and 12% of typical strains of *X. o. oryzae*, respectively, a mixture of two MAbs can be used to reliably detect at least 97% of the typical strains of *X. o. oryzae*.

Mew et al. (1989) were able to isolate *X. o. oryzae* from naturally infected rice panicles and immature grains. In the present study, hulls from approximately two month old rice seed taken from inoculated panicles did not show bacterial streaming although streaming was abundant in the seed-bearing panicle branches as far up as the peduncle. The *X. o. oryzae* also was not detected with IFC or the bacterial streaming test from discolored seed collected from heavily infected cultivar IR-20. On the other hand, the pathogen was detected in high numbers from 0.025 g leaf powder of symptomatic leaves of the same cultivar. The pathogen may not have been present in the seed or numbers were not large enough to form streaming or it may have died during the transit or storage at 4 C. The observation of bacterial streaming in diseased leaves and panicle branches but not in seed, may change our understanding of the seedborne nature of *X. o. oryzae* and its subsequent transmission. If the infected tissue from panicles or leaves is mixed with rice seed from the threshing floor, chances of isolating *X. o. oryzae* should be high only for a short period of time because this pathogen cannot survive for long periods in infected tissue (Hsieh and Buddenhagen, 1975). To date no reports of internal (embryo or cotyledon) seed-borne infection by *X. o. oryzae* have been made.

Dikin et al. (1993) reported detection of *X. o. oryzae* from 8 year old rice seed on XOS medium whereas in the present studies, autoclaved rice seed infested with a typical *X. o. oryzae* strain PXO86 showed 91% reduction in viability of this strain in just 10 months, when seed was stored at 4 C in a desiccator. Atypical fast growing *X. o. oryzae* strains of low virulence (Gnanamanickam et al., 1993; Jones et al., 1989) grow on XOS medium and these may have been the organisms observed by Dikin et al. (1993). These strains have numerous similarities with typical *X. o. oryzae* but also show some consistent differences in standard biochemical, serological, genetic and fatty acid analyses (Jones, et al., 1989) and grow rapidly on XOS medium. Other fast growing non-pathogenic xanthomonads and other yellow-pigmented bacteria also have been isolated previously

(Benedict, et al., 1989., Jones et al, 1989; Gonzalez, 1991; Di, et al., 1991; Gnanamanickam et al, 1994) and in the present study. All these yellow bacteria may confound morphological identity of *X. o. oryzae* and lead to false identification of this organism (Jones et al, 1989; Di, et al., 1991; Gnanamanickam et al, 1994) unless some specific and reliable methods of detection and identification are used.

The rapid appearance of *X. o. oryzae* colonies on medium E followed by detection with IFC using MAbs Xco-2-FITC and Xoo-7-FITC enhances reliability and facilitates immunodetection of low populations and identification of at least 97% of the slow growing typical strains of *X. o. oryzae* from the diverse rice growing areas of the world. Use of this technique can also be extended to epidemiological, ecological and seed transmission studies to better understand this pathogen of greater economic importance.

Table 3.1. Strains used in IFC and to evaluate semi-selective media for the growth and recovery of *Xanthomonas oryzae* pv. *oryzae*.

Strain	Source/reference
Xanthomonas oryzae pv. *oryzae*	
PXO86	Mew et al. (1992)
PXO70	Mew et al. (1992)
PXO35	Mew et al. (1992)
NXO215	Adhikari et al. (1994)
NXO239	Adhikari et al. (1994)
NXO261	Adhikari et al. (1994)
G-13	S. S. Gnanamanickam, India
G-14A	S. S. Gnanamanickam, India
M8327	S. S. Gnanamanickam, India
T-1	Gnanamanickam et al. (1993)
X1-5	Jones et al. (1989)
X1-8	Jones et al. (1989)
XOO[u]	This study
Avirulent xanthomonads	
Xan1[v]	This study
Xan2[w]	This study
Pantoea (Erwinia) herbicola	Gnanamanickam et al. (1994)
Pseudomonas paucimobilis	
PP1[x]	This study
PP2[y]	This study
Pseudomonas putida[z]	This study

[u] Isolated from leaves of rice cultivar IR 20 from India.

[v] Isolated from rice seed of cultivar IR 20 from India; identified by bacteriological test as *X. campestris*; [using Biolog™ this organism was most closely related to *X. campestris* pv. *strelitizia* but was non-pathogencic on *Strelitizia reginae*].

[w] Isolated from seed of IR 1545-39 grown in Hawaii; identified using Biolog™ system and bacteriological tests

[x] Isolated from rice seed of cultivar IR 20 from India; identified using Biolog™ system and bacteriological tests.

[y] Isolated from seed of rice cultivar IR 1545-39 grown in Hawaii; identified using Biolog™ system and bacteriological tests.

[z] Unidentified strain recovered from rice seed was provided by T. W. Mew, Philippines and was identified using Biolog™ and Rapid NFT strips in this study.

Table 3.2. Composition of the media (per liter) used to analyze some of the components of XOS medium for the growth and recovery of *Xanthomonas oryzae* pv. *oryzae*.

	Media									
Components	A	B	C	D	E	F	G	XOS[a]	XOSM1	XOSM2
Peptone	2g	2g	2g	2g	2g	2g	2g	2g	5g	10g
Sucrose	20g	20g	20g	20g	20g	20g	20g	20g	20g	20g
L-Glutamic Acid	5g	5g	5g	5g	5g	5g	5g	5g	5g	5g
Methylviolet-2B	0.3mg	0.3mg	0.3mg	0.3mg	0.3mg	0.3mg	0.3mg	0.3mg	0.3mg	0.3mg
FeEDTA	-	1mg	-	-	1mg	-	1mg	1mg	1mg	1mg
$Ca(NO_3).4H_2O$	-	-	0.2g	-	0.2g	0.2g	-	0.2g	0.2g	0.2g
K_2HPO_4	-	-	-	2g	-	2g	2g	2g	2g	2g
Kasugamycin[a]	-	20mg	20mg	20mg	20mg	20mg	20mg	20mg	20mg	20mg
Cephalexin[a]	-	20mg	20mg	20mg	20mg	20mg	20mg	20mg	20mg	20mg
Cyclohexamide[a]	-	100mg	100mg	100mg	100mg	100mg	100mg	100mg	100mg	100mg
Difco Agar	17g	17g	17g	17g	17g	17g	17g	17g	17g	17g

[a] Di et al. (1991).

Table 3.3. Plating efficiency on XOS agar medium amended with different concentrations of FeEDTA for the recovery of typical and atypical strains of *Xanthomonas oryzae* pv. *oryzae*

	Plating efficiency (%)[x]			
Strain	XOS(1mg)	XOS(50mg)	XOS(100mg)	No. of days for colony formation[y]
Typical				
PXO86	92	NG[z]	NG	5-6
PXO70	65	NG	NG	7-8
PXO35	86	NG	NG	7-8
NXO215	76	NG	NG	7-8
NXO261	72	NG	NG	7
NXO239	87	NG	NG	7
G13	96	NG	NG	6-7
G14A	92	NG	NG	6-7
8327M	92	NG	NG	6-7
Atypical				
X1-5	89	99.18	92.62	3
X1-8	82	83.50	72.77	3
T-1	84	87.66	92.85	3

[x] Plating efficiency = Average (10 replications) colony-forming units from the above media per number of colony-forming units on PSA X 100.

[y] Observed at 40 X magnification on XOS (1mg).

[z] No growth observed.

Table 3.4. Recovery of *Xanthomonas oryzae* pv. *oryzae* strains on modified XOS media

	Strains									
	PXO86		PXO35		NXO215		G14-A		XI-5	
Media	CFU[w]	Days[x]	CFU	Days	CFU	Days	CFU	Days	CFU	Days
A	35.0 d[y]	2	9.2 b	2-3	8.4 b	2-3	7.0 a	2	32.8 b	2
B	30.8 bc	2-3	5.8 a	3-4	8.8 b	2-3	10.4 d	2-3	33.2 b	2
C	26.6 bc	2-3	6.8 ab	5-6	10.6 b	3-4	9.4 cd	3-4	33.0 b	2
D	17.8 a	6-7	NG[z]	8	3.6 a	7-8	8.6 bcd	7-8	15.0 a	3-4
E	28.6 bc	2	6.6 ab	3-4	11.0 b	2-3	7.6 abc	2	30.2 b	2
F	28.2 bc	6-7	9.0 b	7-8	5.2 a	6-7	7.6 abc	5-6	28.8 b	3
G	23.6 ab	5-6	7.2 ab	6-7	1 0.8 b	5	7.8 abc	6-7	31.0 b	3-4
XOS	28.4 bc	5-6	7.6 ab	7-8	9.2 b	7-8	7.2 abc	6-7	30.2 b	3
XOSM1	28.6 bc	5-6	6.2 a	7	10.0 b	7-8	6.0 a	6-7	28.8 b	3
XOSM2	23.6 ab	5-6	NG	8	NG	8	NG	8	31.4 b	3

[w] Colony forming units/ml (X 10^{-7}), average of five replications.

[x] Number of days for formation of colonies visible at 40X magnification.

[y] Column means followed by the same letter are not significantly different ($P = 0.05$).

[z] No growth observed.

Table 3.5. Recovery of *Xanthomonas oryzae* pv. *oryzae* on modified E, E1 and XOS media

	Strains									
	PXO86		PXO35		NXO215		G14-A		XI-5	
Medium	CFU[v]	Days[w]	CFU	Days	CFU	Days	CFU	Days	CFU	Days
E[x]	50 a[z]	2-3	18 a	3-4	13 a	2-3	35 a	2	29 a	2
E1[y]	50 a	2-3	21 a	3	12 a	2-3	33 a	2	30 a	2
XOS	46 a	5-6	17 a	7-8	12 a	7-8	32 a	6-7	27 a	3

[v] Colony forming units/ml (X 10^{-7}), average of five replications.

[w] Number of days for formation of colonies (observed at 40X magnification).

[x] XOS medium without K_2HPO_4.

[y] XOS medium with 0.2g/L K_2HPO_4.

[z] Column means followed by same letter are not significantly different ($P = 0.05$).

Table 3.6. Recovery of *Xanthomonas oryzae* pv. *oryzae* and two common saprophytes of rice seed on modified semi-selective media.

	PXO86		*Pantoea herbicloa*		*Pseudomonas putida*	
	CFU[u]	Days[v]	CFU	Days	CFU	Days
E	53 a[y]	2-3	NG[z]	-	42 a	1
E(T)[w]	50 a	2-3	NG	-	44 a	1
E1	52 a	2-3	NG	-	43 a	1
E1(T)[x]	50 a	2-3	NG	-	47 a	1
XOS	51 a	5-6	NG	-	40 a	1-2

[u] Colony forming units/ml (X 10^7), average of 10 replications.

[v] Number of days for formation of colonies visible at 40X.

[w] Modified with 10 mg/L TZC.

[x] Modified with 10 mg/L TZC.

[y] No growth observed.

[z] Column means followed by same letter are not significantly different ($P = 0.05$).

Table 3.7. Recovery of typical and atypical strains of *Xanthomonas oryzae* pv. *oryzae* on XOS, E, E1 agar media.

	Strains											
	X1-5		X1-8		T1		PXO86		PXO70		PXO35	
Media	Rec.[x]	Days[y]	Rec.	Days	Rec.	Days	Rec.	Days	Rec.	Days	Rec.	Days
E	98 [az]	2	44 [a]	2	69 [a]	2	238 [a]	2-3	87 [b]	2-3	69 [a]	3-4
E1	94 [a]	2	45 [a]	2	66 [a]	2	238 [a]	2-3	78 [a]	2-3	65 [a]	3
XOS	91 [a]	3	45 [a]	3	64 [a]	3	233 [a]	5-6	76 [a]	7-8	60 [a]	7-8

	Strains											
	NXO215		NXO261		NXO239		G13		G14A		8327M	
Media	Rec.	Days	Rec.	Days	Rec.	Days	Rec.	Days	Rec.	Days	Rec.	Days
E	54 [a]	2-3	98 [b]	2-3	121 [b]	2-3	131 [b]	2-3	49 [a]	2	92 [a]	2
E1	54 [a]	2-3	93 [b]	2-3	123 [b]	3	132 [b]	2-3	46 [a]	2	85 [a]	2
XOS	49 [a]	7-8	70 [a]	7	94 [a]	7	96 [a]	6-7	45 [a]	6-7	90 [a]	6-7

[x] Recovery in colony forming units/ml X 10^{-6}. (Average of 10 replications).

[y] Number of days for formation of colonies (observed at 40X magnification).

[z] Column means for each medium followed by the same letter are not significantly different ($P = 0.05$).

Table 3.8. Detection of *Xanthomonas oryzae* pv. *oryzae* strain PXO86 mixed with rice seed extract on medium E[w] (Expt. 1).

	Colony forming units/ml							
	Pour Plate				Spread Plate			
	PXO86		Contaminants		PXO86		Contaminants	
Sample[w]	Exp[x]	Det[y]	Exp	Det	Exp	Det	Exp	Det.
1	1926	TM[z]	208000	TM	1633	TM	182000	TM
2	1926	TM	20800	TM	1633	TM	18200	TM
3	1926	1185	2080	1720	1633	1100	1820	1400
4	1926	1413	208	198	1633	1243	182	170

[w] CFU in 10-fold dilutions of seed extract; a constant number of *X. o. oryzae* was mixed with samples 1 to 4; IFC was performed on all samples in the pour plate medium to identify colonies of *X. o. oryzae*.

[x] Expected number of cfu/ml of *X. o. oryzae* and contaminants determined by plating a suspension of PXO86 and unmixed seed extract, respectively.

[y] Detected number of cfu/ml.

[z] Too many to count by visual observation. *X. o. oryzae* colonies were clearly visible in these samples when examined by IFC.

Table 3.9. Detection of *Xanthomonas oryzae* pv. *oryzae* strain PXO86 mixed with rice seed extract on medium E[W] (Expt. 2).

	Colony forming units/ml							
	Pour Plate				Spread Plate			
	PXO86		Contaminants		PXO86		Contaminants	
Sample[a]	Exp[X]	Det[Y]	Exp	Det	Exp	Det	Exp	Det
1	1553	TM[Z]	187000	TM	1268	TM	171800	TM
2	1553	TM	18700	TM	1268	TM	17180	TM
3	1553	1002	1870	1685	1268	730	1718	1016
4	1553	1205	187	215	1268	973	172	183

[W] Ten-fold dilutions of seed extract; a constant number of *X. o. oryzae* was mixed with samples1 to 4; IFC was performed on all samples in the pour plate method to identify colonies of *X. o. oryzae.*

[X] Expected number of cfu/ml.of *X. o. oryzae* and contaminants determined by plating a suspension of PXO86 and unmixed seed extract, respectively.

[Y] Detected number of cfu/ml.

[Z] Too many to count by visual observation. *X. o. oryzae* colonies were clearly visible in these samples when examined by IFC.

Table 3.10. Detection of *Xanthomonas oryzae* pv. *oryzae* (strain PXO86) on medium E (pour plate) from infested rice seed.

		Colony forming units/ml[w]		
Sample	Infestation (%)[x]	Contaminants	*X. o. oryzae*[y]	Reaction in IFC[z]
1	0	2.6×10^5	none	-
2	1	3.0×10^5	8.0×10^3	+
3	5	3.3×10^5	2.0×10^4	+
4	10	2.7×10^5	7.4×10^4	+
5	100	none	5.1×10^5	+

[w] Observed from 10^{-3} dilutions of samples 1 to 5.

[x] Number of infested seed per 100 seeds.

[y] Presumptive *X. o. oryzae* based on colony morphology after 3 days.

[z] The immunofluorescence colony staining technique was used to identify *X. o. oryzae* from dried agar pieces (approximately 1 cm^2). Positive reactions = one or more colonies showing bright fluorescence.

Table 3.11. Detection of *Xanthomonas oryzae* pv. *oryzae* (strain PXO86) on medium E (pour plate) from infested rice seed.

		Colony forming units/ml[v]		
Sample	Infestation (%)[w]	Contaminants	*X. o. oryzae*[x]	Reaction in IFC[y]
1	0	4.2×10^4	None	-
2	1	2.9×10^4	ND[z]	+
3	1	6.5×10^4	ND	+
4	1	3.1×10^4	ND	+
5	1	5.3×10^4	ND	+
6	1	3.8×10^4	ND	+
7	5	2.3×10^4	ND	+
8	100	None	4.4×10^4	+

[v] Observed from 10^{-3} dilutions of samples 1 to 8.

[w] Number of infested seed per 100 seeds.

[x] Presumptive *X. o. oryzae* based on colony morphology after 3 days.

[y] The immunofluorescence colony staining technique was used to identify *X. o. oryzae* from dried agar pieces (approximately 1 cm^2). Positive reactions = one or more colonies showed strong fluorescence.

[z] Not determined because of crowding by contaminants on these plates.

Table 3.12. Detection of *Xanthomonas oryzae* pv. *oryzae* from rice seed contaminated with infected leaf tissue[x].

	Observed colony forming/ml					
	Seed extract alone		Leaf tissue		Seed extract+Leaf tissue	
Dilutions	*X. o. oryzae*	Contaminants	*X. o. oryzae*	Contaminants	*X. o. oryzae*	Contaminants
10^{-1}	None	TM[y]	TM*[z]	Few	TM*	TM
10^{-2}	None	TM	TM*	Few	TM*	TM
10^{-3}	None	1.3×10^3	7.4×10^2*	None	2.8×10^2*	7.2×10^2

[x] Detection on medium E (pour plate). Immunofluorescence colony staining (IFC) was performed on the samples from all dilutions.

[y] Too many to count.

[z] Asterisks indicate that samples were positive by IFC.

Table 3.13. Detection of *Xanthomonas oryzae* pv. *oryzae* from rice seed contaminated with infected leaf tissue using medium E and XOS[x].

	Observed colony forming units/ml					
	Seed extract alone		Leaf tissue alone		Seed extract+Leaf tissue	
Dilutions	*X. o. oryzae*	Contaminants	*X. o. oryzae*	Contaminants	*X. o. oryzae*	Contaminants
Medium E						
10^{-1}	None	TM[y]	TM*[z]	Few	TM*	TM
10^{-2}	None	TM	TM*	Few	TM*	TM
10^{-3}	None	7.6×10^2	9.8×10^2*	None	3.2×10^2*	6.8×10^2
XOS						
10^{-1}	None	TM	None	Few	None	TM
10^{-2}	None	TM	None	Few	None	TM
10^{-3}	None	6.7×10^2	None	None	None	6.1×10^2

[x] Detection on pour plate media E and XOS. IFC was performed on the samples from all dilutions.
[y] Too many to count.
[z] Asterisks indicate that samples were positive by IFC.

Table 3.14. Identification of bacteria associated with rice seed infected with symptomatic leaf tissue tissue by direct immunofluorescence and pathogenicity tests on rice cultivar IR-20.

	Identification with Biolog[v]	Colonies tested[w]	IF[x]	Pathogenicity[y]
Colony type 1	*Xanthomonas oryzae* pv. *oryzae*	15	+	+
Colony type 2	*Xanthomonas campestris* [z]	3	-	-
Colony type 3	*Pseudomonas paucimobilis*	2	-	-

[v] Single colony was tested by Biolog™.

[w] Suspected colonies were streaked on YDC for IF and pathogenicity.

[x] Direct immunofluorescence on these colonies was performed: + = strong fluorescence under UV light; - = no fluorescence observed.

[y] + = produced leaf blight symptoms; - = no symptoms produced.

[z] By Biolog™ identified *X. c. strelitizia* but was non-pathogenic on *Strelitizia reginae*. They had bacteriological, physiological and serological characteristics of *X. campestris*

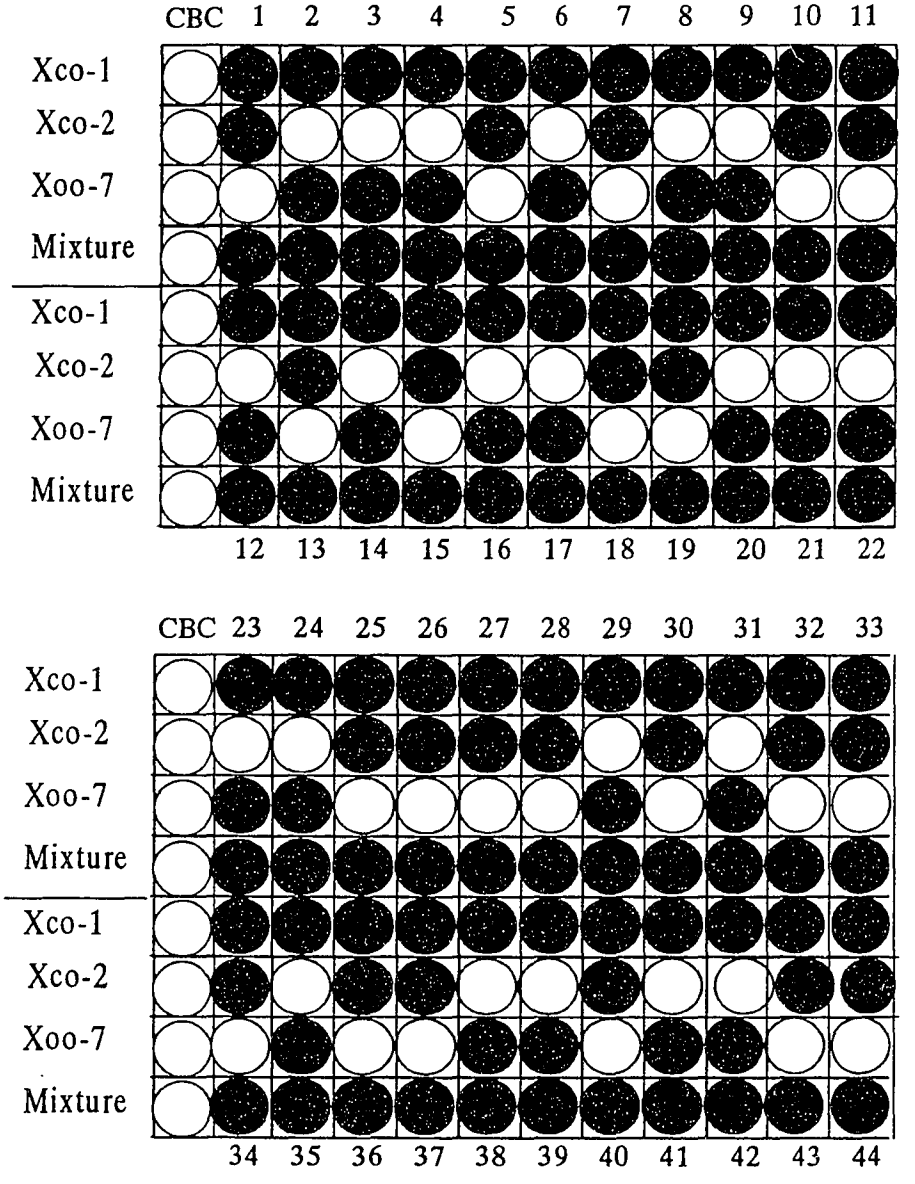

Fig. 3.1. Depiction of ELISA reaction of pathovar specific monoclonal antibodies Xco-1, Xco-2, Xoo-7 and a mixture of Xco-2 and Xoo-7 with 44 randomly picked colonies from 1:1 mixed cultures of *X. o. oryzae* strains PXO86 and PXO35 grown on PSA. The first well in each row contained carbonate-bicarbonate (CBC) buffer (control). Dark wells indicate positive reactions. Wells positive with MAb Xco-2 were negative with MAb Xoo-7, whereas a mixture of the two MAbs was positive in all wells.

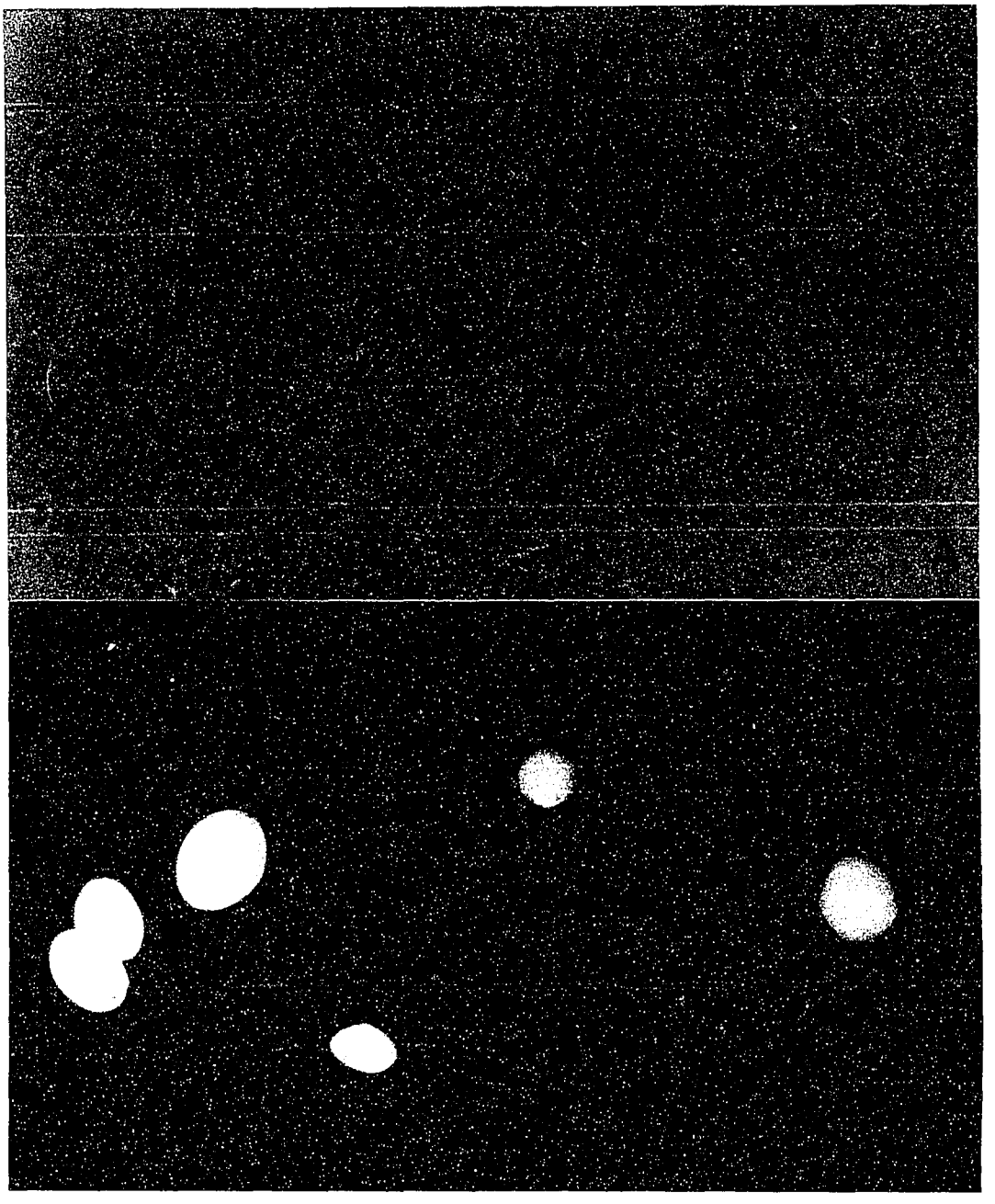

Fig. 3.2. Immunofluorescence colony staining of 1:1 mixed cultures of *Xanthomonas oryzae* pv. *oryzae* strains, PXO35 and PXO86 grown in PSA for 96 hr. An agar piece (approximately 1 $cm^{2)}$ was first stained for 12 hr with 1:50 dilution of a MAb, Xoo-7-FITC homologous to PXO35, **A,** colonies of both strains as observed under transmitted light; **B,** only colonies of the homologous strain PXO35 show bright fluorescence under UV light. The two large colonies, seen in A, of the heterologous strain are not fluorescencing (200X).

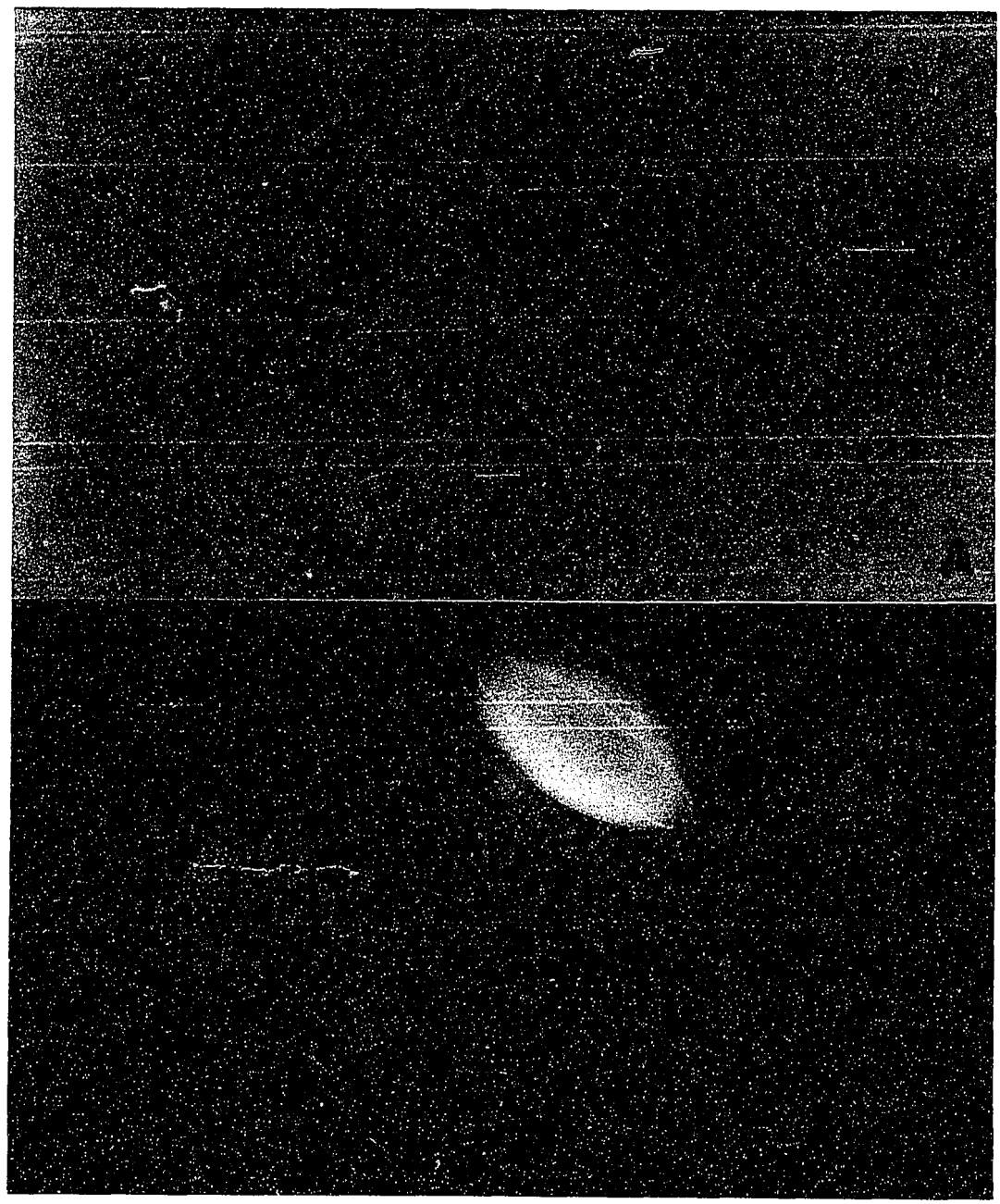

Fig. 3.3. Immunofluorescence colony staining of 1:1 mixed cultures of *Xanthomonas oryzae* pv. *oryzae* strains PXO35 and PXO86. The same agar piece as in Figure 2 was subsequently stained with MAb Xco-2-FITC homologous to PXO86 for another 12 hr. **A**, colonies as observed by transmitted light; **B**, the two large colonies (PXO86) show fluorescence. The bulging margin of one large colony indicates colony growth during the 12 hr staining procedure with second MAb (200X).

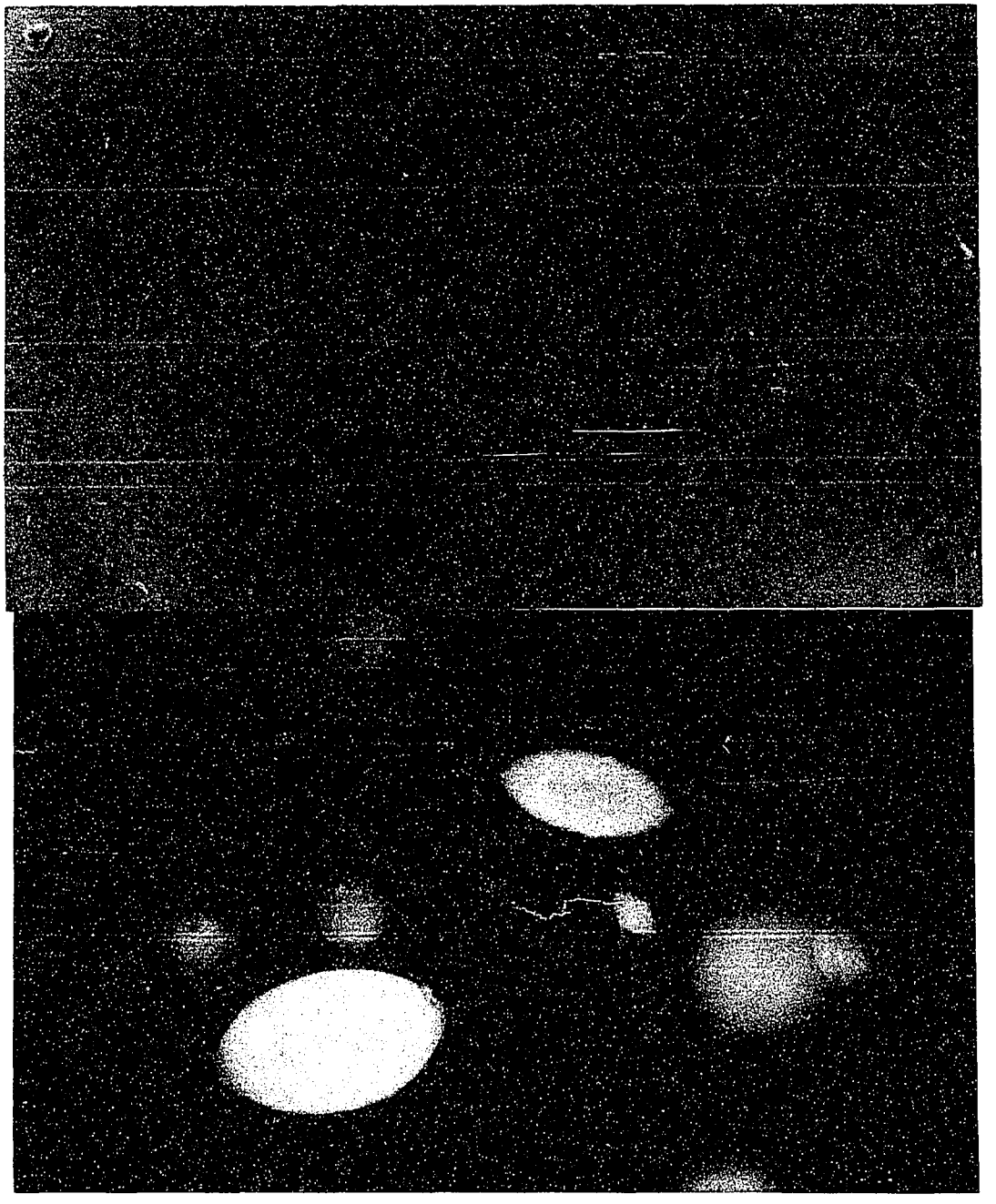

Fig. 3.4. Immunofluorescence colony staining of 1:1 mixed cultures of *Xanthomonas oryzae* pv. *oryzae* strains, PXO35 and PXO86 grown for 96 hr in PSA. Agar pieces (approx. 1 $cm^{2)}$ were stained with a 1:25 dilution of 1:1 mixture of MAbs Xco-2-FITC and Xoo-7-FITC for 12 hr. **A**, colonies of both strains as appeared under transmitted light; **B**, with a mixture of MAbs every colony is showing bright fluorescence under UV light (200X).

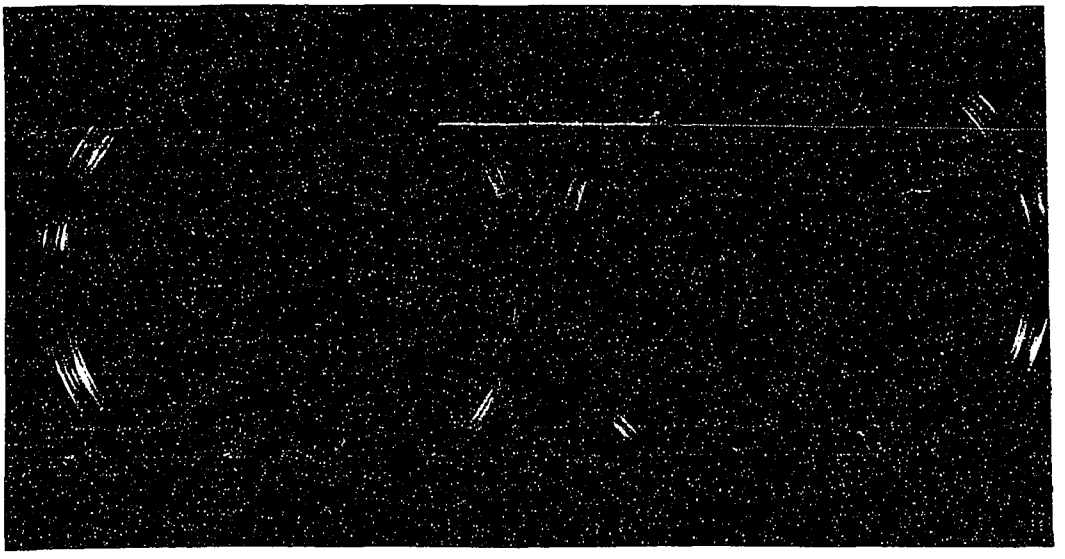

Fig. 3.5. The colony morphology of *Xanthomonas oryzae* pv. *oryzae* strain PXO86 on day 5. **Left**, medium E (without K_2HPO_4); **Right**, medium E1 (containing 0.2 g/L K_2HPO_4).

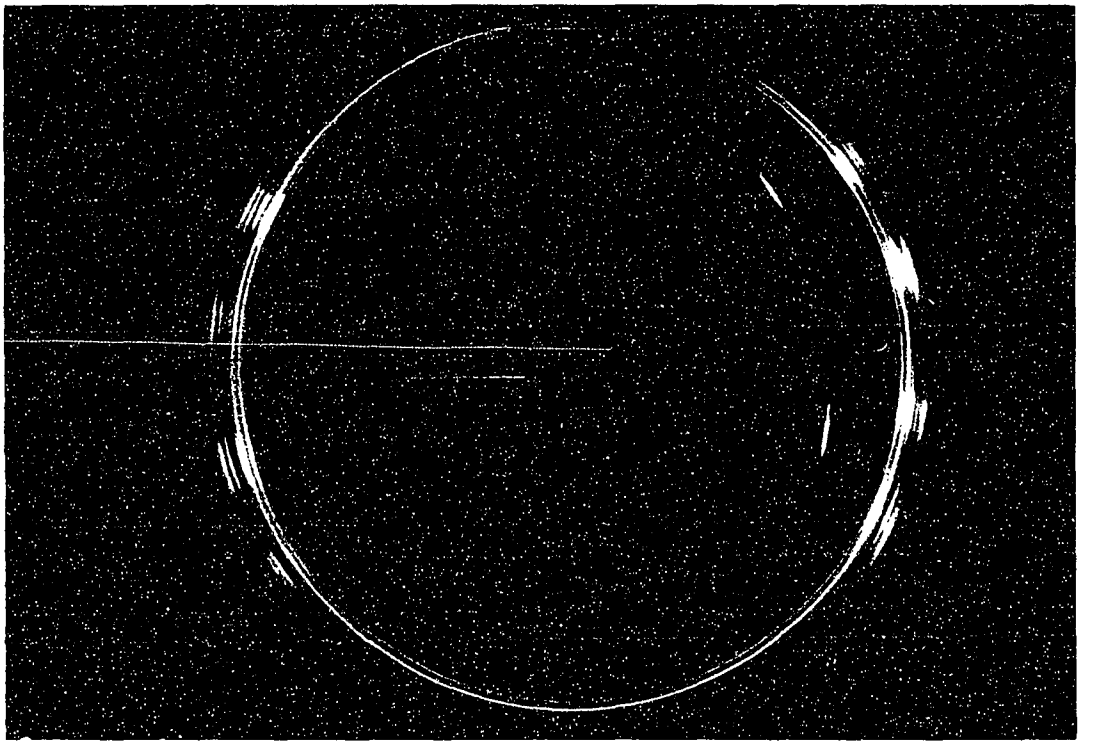

Fig. 3.6. The zone of inhibition produced by *Pseudomonas putida* against *Xanthomonas oryzae* pv. *oryzae* strain PXO86 observed on day 4 on PSA medium.

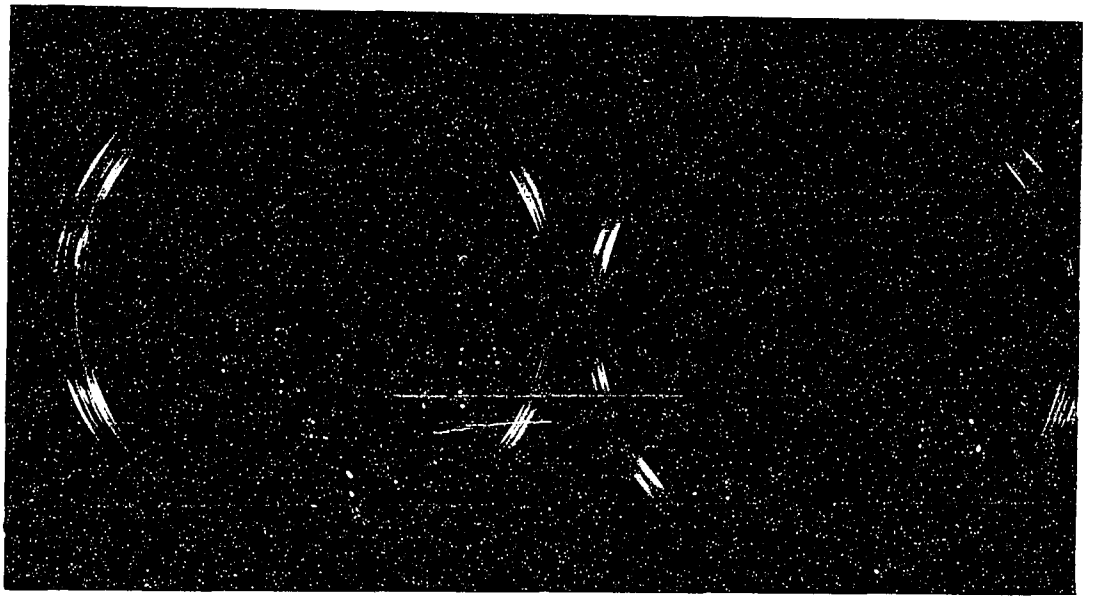

Fig. 3.7. Mixed cultures of *Pseudomonas putida* and *Xanthomonas oryzae* pv. *oryzae* strains PXO86 on day 7. Left, medium E (without K_2HPO_4); Right, medium E1 (0.2 g/L K_2HPO_4). Colony diameters *P. putida* are smaller on medium E compared to medium E1 and more colonies of *X. o. oryzae* also are present on medium E than medium E1. Arrows point to *X. o. oryzae* colonies.

Fig. 3.8. Colonies of rice seed contaminants and *Xanthomonas oryzae* pv. *oryzae* strain PXO86 grown for three days on medium E. The arrow points to a very small single colony of *X. o. oryzae* visible among many contaminants. The differential sizes of the *X. o. oryzae* and contaminant colonies aided in visual detection of target colonies (60X).

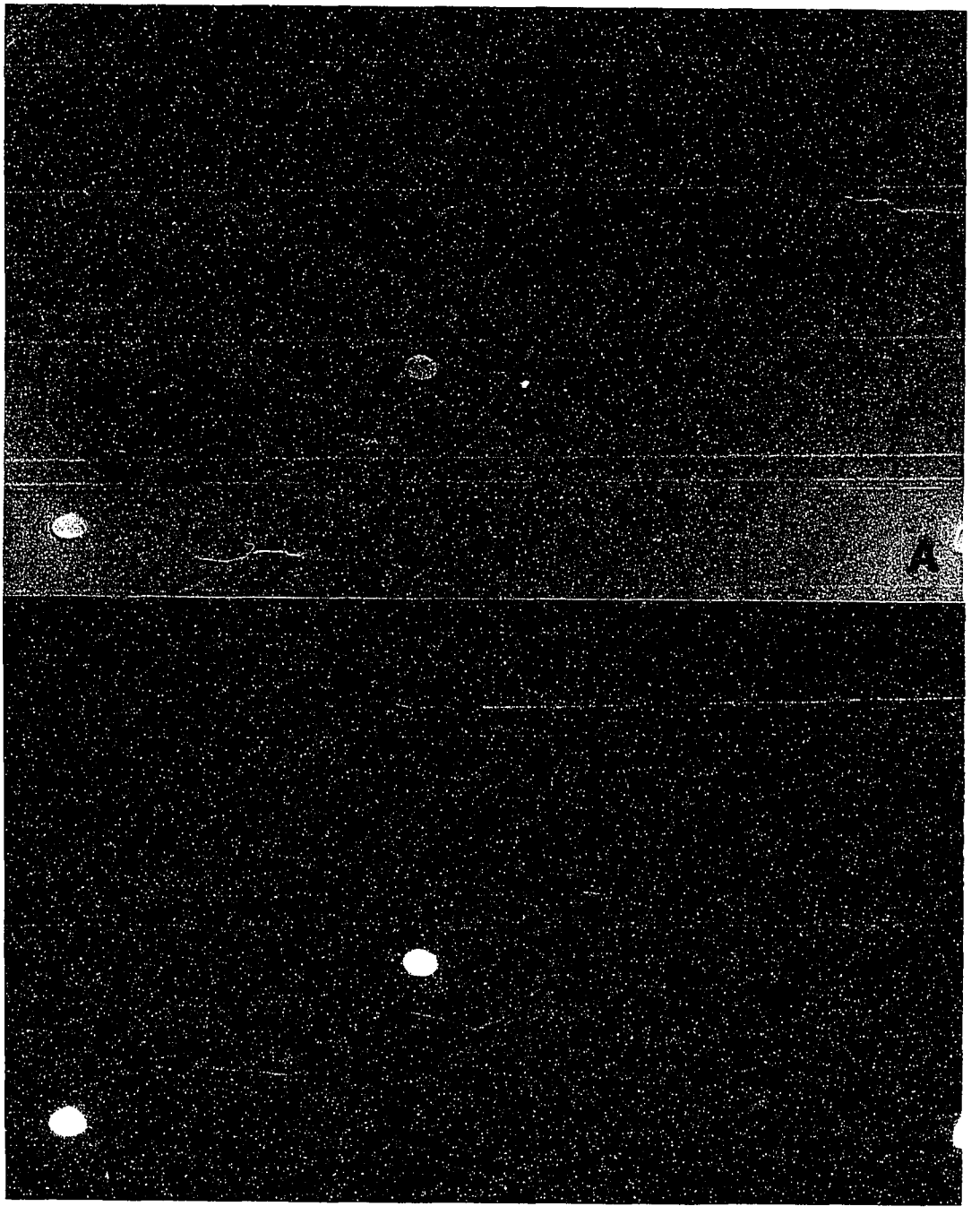

Fig. 3.9. Colonies of rice seed contaminants and *Xanthomonas oryzae* pv. *oryzae* strain PXO86 grown for three days on medium E and treated with MAb Xco-2-FITC. A, colony appearance in 50% transmitted and 50% UV light. Two large contaminant colonies and three fluorescent colonies *X. o. oryzae*; B, colonies of *X. o. oryzae* giving bright fluorescence under UV light (170X).

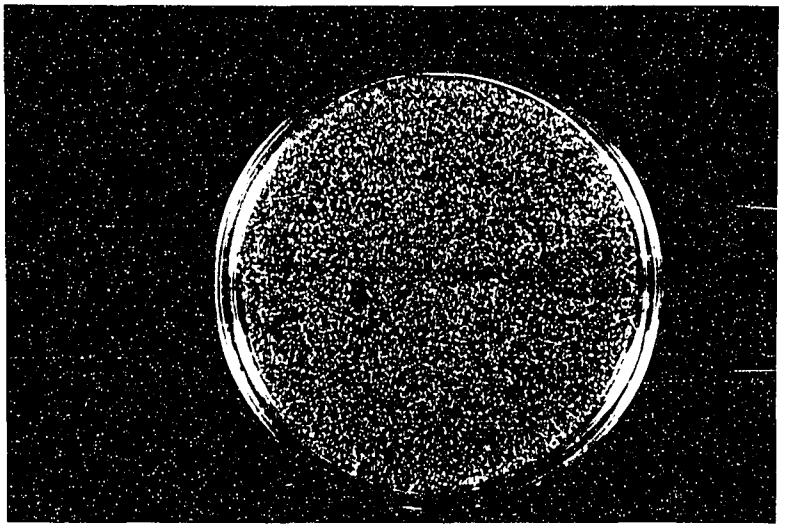

Fig 3.10. Colonies of rice seed contaminants formed on medium E four days after plating. Five artificially infested seeds (4.4×10^2 cfu/seed) were mixed with 95 healthy seeds, soaked in 5 ml sterile saline, mixed with 12 ml molten agar and plated. Eighty seven *Xanthomonas oryzae* pv. *oryza*e were detected on this plate by immunofluorescence colony staining technique.

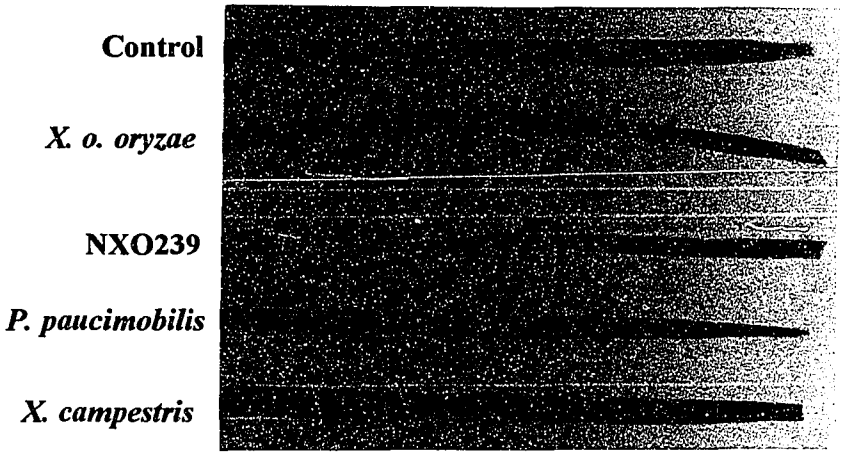

Fig 3.11. Symptoms produced on rice cultivar IR-20 by a known *Xanthomonas oryzae* pv. *oryza*e (NXO239) and a presumptive *X. o. oryza*e recovered from naturally infected rice leaf tissue. An unidentified *X. campestris* strain and a *Pseudomonas paucimobilis* strain recovered from rice seed extract were also tested. Sterile saline served as a control

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