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Novel Strains of *Xanthomonas oryzae* pv. *oryzae* UV Mutated Induce Systemic Resistance in Rice against Bacterial Leaf Blight Disease

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ABSTRACT

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is the most devastating bacterial disease of rice production worldwide. Studies were conducted to develop management strategies using attenuated Xoo strains that were nearly or equally effective with previous ISR-P® uses (*Pseudomonas fluorescens* SP007s product). Bacterial suspension of the virulent wildtype strain Xoo-19 was placed on nutrient agar and exposed to ultraviolet (UV) radiation (65 W) for 3 min. Two UV-induced biotype strains (M-407 and M-690) that survived the irradiation and reduced the growth rate and pathogenicity were selected and characterized. They differed from the wildtype Xoo-19 in colony morphology, production of bacteriocin and exopolysaccharide, enzyme activity (cellulase and protease), and reduced motility on the surface of agar. They were investigated further for their ability to control BLB and activate defense-related enzymes, β-1,3-glucanase and peroxidases (POX) under greenhouse conditions, compared with a naturally-occurring avirulent strain of Xoo obtained in this study (Xoo-7) and the strain SP007s. Twenty one-day old rice plants of two cultivars (susceptible cv. Khao Dauk Mali105 or KDML105 and resistant cv. Suphanburi 1) separately pretreated with 1×10^8 cfu/mL of each of four antagonist strains as foliar sprays, were inoculated with the wildtype strain Xoo-19 at 3 d after pretreatment. All four strains, namely M 690, M-407, Xoo-7 and SP007s, provided significant (P = 0.05) disease reduction by 53.7, 49.5, 45.2, and 58.1% with KDML105 and 62.1, 51.4, 47.9, and 62.1% with Suphanburi 1, respectively, when assessed 14 d post inoculation compared to the nontreated control. Strain M-690 was equal to or nearly as effective in disease reduction as SP007s with no significant (P = 0.05) difference between both cultivars tested. The M-690 and SP007s strains also showed the highest accumulation of β-1,3-glucanase and POX activity in rice plants that correlated with their disease reduction ability. Control efficacy of all four antagonist strains showed a difference in the two-rice cultivars, with Suphanburi 1 being higher than KDML105 in both disease reduction and defense-related enzyme accumulation.

Keywords: biocontrol, UV mutants, naturally-occurring avirulent strain, activated plant defense, defense related enzyme

INTRODUCTION

Rice is one of the most important staple foods for the increasing world population, especially in Asia. More than 70 diseases caused by fungi, bacteria, viruses and nematodes are among the most important limiting factors that affect rice production (Song and Goodman, 2001).

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The bacterial leaf blight disease (BLB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is one of the most serious constraints to high rice production (Ou, 1985). Severe BLB results in yield loss upon premature plant death or lodging of infected necks. Disease management with the use of resistant cultivars is considered to be effective in minimizing the damage, but rice cultivars resistant to BLB are limited. In addition, resistant cultivars with one or two major resistant genes are unsustainable in the field because of the high pathogenic variability of Xoo under field conditions (Babu *et al.*, 2003). Several broad spectrum fungicides and bactericides have been recommended for the control of BLB. However, they are expensive and can affect the beneficial microbial population present in the ecosystem leading to obvious pollution problems (Kagale *et al.*, 2004).

In recent years, there has been increasing interest in the biological control of bacterial plant diseases using either plant growth promoting rhizobacteria (PGPR) or naturally-occurring saprophytic bacteria (Hsieh and Buddenhagen, 1974). Antagonistic interaction between closely related strains of both Gram-negative and Gram-positive bacteria is often influenced by the production of bacterial toxins, termed bacteriocins (Dardick *et al.*, 2003). Sakthivel and Mew (1991) showed the potential control efficacy of nonpathogenic mutants of bacteriocin-producing strains against bacteriocin-sensitive challenged strains. However, antagonism between strains of plant pathogenic bacteria is not limited to the secretion of bacteriocins (Dardick *et al.*, 2003). The control efficacy of several *hrp* mutants from *Erwinia, Pseudomonas* and *Xanthomonas* against parental strains by co-inoculation or challenge-inoculation have been described, where the mechanisms of inhibition by these mutants remain unclear (Moss *et al.*, 2007).

Investigations on mechanisms of disease suppression by biological control agents, including *Bacillus amyloliquefaciens* (KPS46) and *Pseudomonas fluorescens* (SP007s), have been well documented (Chuaboon, 2005; Preecha *et al.*, 2010). However, little is known about disease suppression facilitated by attenuated mutants of Xoo against subsequent infections with their parental strains. The active principles may act on the pathogen either directly or indirectly through induced systemic resistance (ISR) in host plants resulting in the reduction of disease development. ISR activates multiple defense mechanisms, including increased activity of pathogenesis-related (PR) proteins like chitinase, β-1,3-glucanase and peroxidases (POX), as well as the accumulation of phytoalexins (Kagale *et al.*, 2004). Evaluation of biological control agents against various diseases of economic crops on the ISR mechanism has been attempted under both greenhouse and field conditions (Prathuangwong and Buensanteai, 2006). However, no attempts have been made to understand the mechanisms of disease resistance induced by attenuated mutants of Xoo against challenge-innoculation with their parental strains. Therefore, this study aimed to evaluate the potential for the induction of systemic resistance in rice by attenuated UV-mutant strains compared with the naturally-occurring avirulent strains, Xoo-7 and SP007s.

MATERIALS AND METHODS

Bacterial strains and culture condition

Bacterial strains used in the present study were isolated from different rice-growing regions in Thailand by the leaf-grinding technique of infected tissues (Ghasemie *et al.,* 2008). The purified strains were subcultured on nutrient agar (NA) for routine use and stored in 20% glycerol at -80°C for long-term storage. The bacterial suspension at 10^8 cfu/mL were used to innoculate the susceptible cv. Khao Dauk Mali 105 (KDML105) for the pathogenicity test, as described by Kauffman *et al.* (1973) and to

infiltrate the tobacco (*Nicotiana tabaccum*) and tomato leaves for the hypersensitive response (HR) test. Disease induction was observed every day, symptom development was recorded and final lesion length was measured 14 d after inoculation. Strains were considered pathogenic if more than 60-70% of the inoculated plants showed lesion development of BLB. Infected plants were monitored for 2-3 d before the causal agents were reisolated to fulfill Koch's postulate.

Identification of *X. oryzae* pv. *oryzae*

Bacterial strains that had expressed lesion development after pathogenicity testing were identified tentatively using standard morphological characteristics, followed by 16S-23S rDNA-PCR analysis. Oxidative or fermentative metabolism, including biochemical characteristics and information from Bergey's Manual, was used for a comparative study (Hung and Leifsun, 1953; Schaad, 1980). Strains already tested for phenotype identification were then cultured for DNA extraction (Adachi and Oku, 2000). The primers used for polymerase chain reaction (PCR) amplification were the 16S-23S intergenic region of the specific primer, XOR-F;5′-GCATGACGTCATCGTCCTGT-3′ and XOR-R2; 5′-CTCGGAGCTATATGCCGTGC-3′, respectively, as described by Adachi and Oku (2000), with minor modification. PCR amplification was carried out with a thermocycler (MyGenie 32, Global Genomic Partner). The PCR program was edited according to the following cycles: initial denaturation at 95°C for 7 min, followed by 35 cycles including denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 6°C for 8 min and the final extension at 72°C for 15 min, followed by final incubation at 4°C. An amount of 10 μL from each amplified PCR product was taken, loaded and run on a 0.75% agarose gel in 1×TBE buffer at 50 V and 37 A for 2 h and stained with ethidium bromide and photographed with a UV transilluminator. A 100-bp DNA ladder (molecular marker) was used for molecular size reference of the bands of each strain yielded from PCR analysis, as described by Adachi and Oku (2000).

The UV-induction and isolation of mutants

To attenuate the target strain by UV-mutagenesis, the test strain which showed the most aggression (symptoms development at shorter incubation period and longer lesion length at 14 d after inoculation) was grown overnight in nutrient broth (NB) medium. The bacterial suspension of 0.2 at OD_{600nm} (10^8cfu/mL) was plated onto NA medium. Petri dishes with the lids removed were exposed to UV irradiation (65 W) for 1, 2, 3, 4, 5, 10, and 15 min at constant distance from the UV light source (300 mm apart) as modified by Preecha *et al*. (2010). The UV-irradiated Petri dishes were incubated at 28 ± 3°C in the dark until surviving colonies were visible. Colonies from Petri dishes containing about 10-20% survivors were again plated onto NA medium and exposed twice to UV irradiation. Colonies from the second irradiation were transferred to 'master' dishes with sterile toothpicks to give an array of 50 colonies per dish and were subsequently examined for their colony morphology. Only colonies with altered morphology were selected for examination of growth rate in NB medium and swimming motility in the 0.4% agar medium. They were chosen finally to examine their pathogenicity on susceptible rice cv. KDML105 by detached leaf assay (Akhtar *et al*., 2008).

Screening and characterization of non-pathogenic mutants

After colony morphology and motility assays, the defective pathogenicity of the Xoo mutant and naturally-occurring avirulent strains was determined by inoculating them onto KDML105 and comparison with the wildtype by detached leaf assay (Akhtar *et al*., 2008). Briefly, the leaves of plants aged 30-40 d were washed

throughly under running tap water to remove dirt and then surface-disinfected in 70% ethanol and rinsed twice with sterile distilled water. The leaves were placed adaxial side upward on four layers of sterile paper towel on two glass slides in 90 mm Petri dishes. The leaves were then mounted by pin point to make the wounds and 10 µL of bacterial suspension at a concentration of approximately 10^8 cfu/mL was loaded onto the wounds. After inoculation, five leaves were kept in each Petri dish. Similarly, the leaves were inoculated by dipping the inoculating scissors in each inoculum kept in a beaker. The leaves were incubated under controlled conditions (96% RH under 28 ± 3°C). Symptom development was compared with the wildtype and distilled water was used as the negative control. Disease induction was observed at 3, 6, 9, 12, 24, 48 and 168 h and the length of lesions or colonization based on evidence of an enlarged bright field around the wounds were measured in centimeters after 14 d of inoculation.

Bacteriocin production and inhibition of wildtype

Bacteriocin production of mutants was evaluated according to the methods described by Bonini *et al*. (2007). The strains were transferred to Petri dishes containing yeast peptone dextrose agar (YPDA) medium (0.6 g peptone, 3 g dextrose, 3 g yeast extract, 15 g agar, and 1000 mL distilled water). After growth at 28 ± 3°C for 24 h, the colonies were transferred to other Petri dishes containing YPDA medium with a nine-felt-discs dispenser (4 mm in diameter), with each disc corresponding to one strain. After growth of the strains on the culture surface (28 ± 3°C, 24 h), the Petri dishes were placed in an inverted position under an exhaust hood, 1 mL of chloroform was added to the lid of each dish and the dishes were incubated for 2 h for bacterial inactivation. For the determination of bacteriocin production, the dishes were overlaid with 5 mL semi-solid melting (45°C) YPDA medium containing 1 mL each of bacterial suspension previously cultured separately in liquid YPD medium. The Petri dishes were incubated for 24 h at 28 ± 3°C and the presence or absence of an inhibition halo around the bacteriocin-producing colonies was recorded. From these results, mutant strains which showed the highest production of bacteriocin were selected to suppress the production of the sensitive strain of bacteriocin by dual culture and the poisoning medium technique.

Development of tolerance to antibiotics and growth in planta

The bacterial growth in broth was compared and the bacterial population density of each bacterial strain was measured 0, 6, 9, 12, 24, 48, and 72 h after incubation using a spectrophotometer. The investigation on the growth rate of bacteria in planta was made by detached leaf assay in a moist chamber, as described above. Each selected strain was macerated and placed individually in sterile distilled water, triturated and isolated by plating to form individual colonies on NA medium containing the appropriate antibiotics, rifampicin, ampicillin, and streptomycin separated at different concentrations (12.5, 25, 50, 75, 100 mg/L) for Xoo-19, M-690, and M-407 resistant marker tested. The reduction in the population of the wildtype strain in vitro and in planta was investigated by mixed-culture strains in different ratios (1:1, 1:2, 1:4, 1:5, 1:10, 1:50) of mutant:wildtype in NB broth and the population density was determined, as described above. The suppression of the wildtype population and disease reduction at different ratios in mixed inoculation on the rice leaves was also investigated (Dardick *et al*., 2003).

Assays on extracellular enzyme and exopolysaccharide production

Relative levels of exoenzyme production were assessed by radial diffusion assays. The wildtype and UV mutants were grown overnight in nutrient yeast glucose broth (NYGB) at 28 ± 3 °C. Cells were pelleted by centrifugation and the

supernatants were collected and sterilized by filtration (0.2 μm). Then, 30 μL of the filter-sterilized supernatants was placed in a 0.5-cm well made in an agar medium using a cork borer, especially for cellulase, protease, and α-amylase activity. The ability of the wildtype and mutant to cause potato tuber maceration and the production of extracellular polysaccharide was also determined (Thowthampitak *et al*., 2008). Growth on peptone sucrose agar (PSA) medium, colony morphology, and motility of mutants and the wildtype were also compared (Jeong *et al*., 2008).

In planta experiment for biological control effectiveness

Rice seedlings (cvs KDML105 and Suphanburi 1) were grown in the greenhouse, as described previously. The selected bacterial strains (M-690, M-407, naturally-occurring avirulent strain of Xoo (Xoo-7), and SP007s) were grown on NA medium for 24 h at 28 ± 3°C and bacterial cultures were suspended in NB and incubated overnight on a rotary shaker at 150 rpm. The bacterial suspension was adjusted spectrophotometrically to the appropriate concentration at 0.2 OD_{600nm} (10^8 cfu/mL). The control efficacy of mutants and induction of plant defense-associated enzymes in leaves of KDML105 and Suphanburi 1 were assessed in 21 day-old rice plants that were: 1) pre-treated with M-690, M-407, Xoo-7 and SP007s separately at 3 d prior to inoculation with Xoo-19; and 2) pre-treated with sterile water and inoculated with Xoo-19. The experiment was conducted two times and a completely randomized design was used in all experiments.

Scoring of BLB disease and disease suppression by strains M-407, M-690, Xoo-7 and SP007s were investigated 14 d after inoculation with the wildtype. The diseased lesion area and the entire leaves were measured. Disease scoring was carried out based on the average area of diseased lesion per whole leaf area (L/W). A disease scale with a maximum score of 9 and a minimum score of 0 was used, with score 0 indicating no infection of leaves, so L/W = 0; score 1, L/W < 0.2; score 3, L/W = 0.25; score 5, L/W = 0.5; score 7, L/W = 0.75; and score 9 = complete death of leaves. A disease index (DI) was calculated using Equation 1:

DI = 100 × Sum of individual scores / Total leaves observed × Maximum score (1)

The extent of disease reduction attributed to each treatment was calculated using Equation 2, as modified from Ji *et al*.(2008):

Disease suppression efficiency = (Disease index of control – Disease index of treatment group) / (Disease index of control) x 100% (2)

Analysis of variance (ANOVA) for disease index and suppression efficacy was performed using the IRRISTAT version 5.0 software. Mean comparisons were conducted using a least significant difference (Fisher's LSD) test (P = 0.05 or P = 0.01). The standard error and LSD results were recorded. Calculation of percentage disease suppression for the biological control treatment with different biocontrol agents allowed a mean disease reduction to be calculated across replicate experiments. The experiment was repeated twice.

Defense-related enzymes induced by mutants

Rice plants treated with bacterial strains and distilled water and challenge-inoculated with the pathogenic wildtype were investigated, as described above. Leaves were collected first prior to challenge-inoculation with the pathogen and a subsequent sample was collected every day starting at day 1 until 7 d after pathogen inoculation. Three independent samples were collected and assayed for changes in defense-related enzymes, including β-1,3-glucanase and peroxidases (POX). To determine the response during plant, mutant and pathogen interaction, leaf samples (0.1 g weight of tissue) were ground in a mortar and pestle containing extraction buffer (0.1

M Tris-HCl buffer, pH 7, 0.1 M KCl, 1 mM phenylmethanesulfonyl fluoride, 10 mL/L Triton X-100, 30g/L polyvinylpyrrolidoneK30). The homogenate was centrifuged at 12,000 rpm at 4° C for 10 min and the supernatant was kept on ice until the enzyme activity assay. The total protein concentration in the extracts was measured with a modified assay described by Bradford (1976), in which 1 mL of Bradford reagent was added to 0.1 mL of extracts and the absorbance of the mixture was read at 595 nm after a reaction time of 2 min. The sample protein content was determined from a standard curve generated with bovine serum albumin. The activities of POX and β-1,3-glucanase were determined according to Hammerschmidt *et al*. (1982) and Pan *et al*. (1991) respectively. Each reaction was started by adding standard reagent to the mixture, including hydrogen peroxide and laminarin for the POX and β-1,3-glucanase assays, respectively. The enzymatic activity was expressed as units/mg enzyme, (µg POX, μg glucose and catechol/µg, protein, respectively. The reaction mixture was incubated in a water bath at 35°C for 15-20 min and the absorbance reading at a suitable wavelength (290-725 nm) was determined before and after incubation to calculate the increase in absorbance.

RESULTS AND DISCUSSION

Isolation and characterization of *X. oryzae* pv. *oryzae*

Twenty seven strains of Xoo collected from different rice growing regions were investigated for their morphology and physiology. They were Gram-negative, rod-shaped, round-ended and tested negative for oxidase, catalase, starch hydrolysis and tetrazolium tolerance. Acid production was observed from glucose, fructose and galactose, but not from ribose, maltose and lactose. All strains tested positive with the gelatin hydrolysis test. Colonies on solid media containing sucrose were round, convex, mucoid and yellow in color, due to the production of the pigment xanthomonadin, the characteristic of the genus, as described by Shen and Ronald (2002). All isolated Xoo strains showed necrotic lesions on nonhost plants at the infiltration point within 24-48 h post inoculation, according to the HR test (Figure 1).

All tested strains caused leaf blight on the surface of rice leaves 2 w after inoculation, although their incubation period for the first symptoms initiated and disease severity were different. Symptoms of BLB appeared on leaves as pale-green to grey-green water-soaked streaks near the leaf tip and margin. These lesions coalesced and became yellowish-white with wavy edges. Leaf sheaths and culms were attacked (Figure 2). These symptoms did not occur in the control plants. Differences in virulence among the

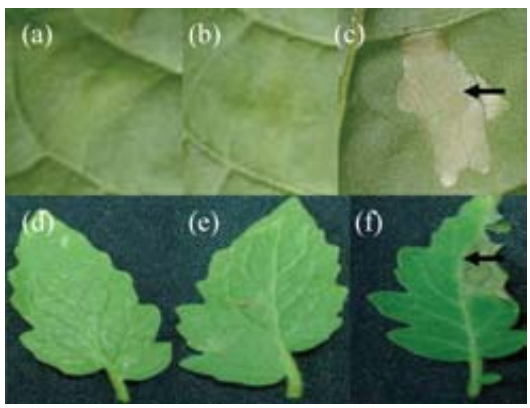

Figure 1 Hypersensitive response (HR) elicited on tobacco leaves ((a), (b) and (c)) and tomato leaves ((d), (e) and (f)) by *Xanthomonas oryzae* pv. *oryzae* strain Xoo-19 wildtype (c) and (f), but not by strains M-690 (a) and (d) and M-407 UV mutant (b) and (e), respectively. Leaves were infiltrated to the right of the midvein using the strains indicated. The lateral veins forming the upper and lower borders of the necrotic zone on the wildtype infiltrated leaves are indicated by black arrows.

Xoo strains collected were quantified according to the lesion length of the necrotic area and the incubation period. The time at which BLB symptoms occurred is also an important indicator of virulent pathogen strains, as Xoo-19 was shown to cause symptoms at day 4 after inoculation. It also showed the earliest development of disease and the highest lesion length at 14 d post inoculation. Assays of strain Xoo-19 in either susceptible or resistant cvs of rice plants displayed the same pattern of virulence (Figure 2). Strain Xoo-7 showed the lowest lesion length at day 7 of the incubation period and failed to enlarge the lesion length (data not shown). These two strains were chosen as virulent and non-virulent pathogens, respectively, for further investigation.

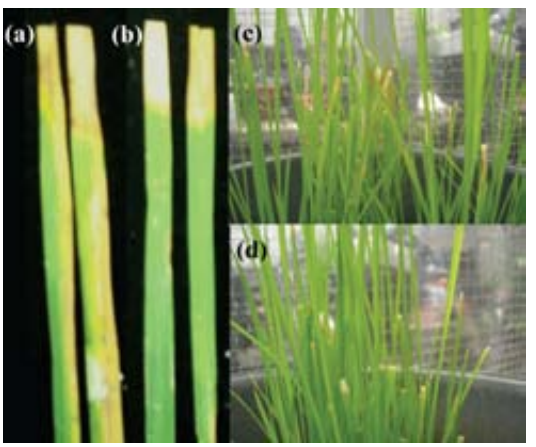

Figure 2 Development of bacterial leaf blight symptoms in rice plants inoculated with *Xanthomonas oryzae* pv. *oryzae* strain Xoo-19 wildtype. Lesions on susceptible cv. KDML105 (a) and resistant cv. Suphanburi 1 (b), respectively, throughout 21 d post inoculation by detached leaf assay. Leaf sheaths and culms turned yellowish white under greenhouse inoculation with the Xoo-19 wildtype on cv. KDML105 (c) and cv. Suphanburi 1 (d), respectively.

Molecular identification of causal pathogen

The causal agent of the BLB pathogen was confirmed as Xoo by 16S-23S rDNA and PCR analysis. All strains of Xoo studied were developed with a conservative region of 16S-rDNA amplification. A 470-bp product was amplified successfully for all Xoo strains tested (Figure 3). Each analysis of the amplification products of Xoo strains showed the presence of one similar product indicating the primer use was complementary to the intergenic region between 16S and 23S rDNA. This intergenic region primer was specific for Xoo and was found to be capable of detecting all strains producing BLB symptoms tested in vivo (Adachi and Oku, 2000). These primers should be a powerful tool to detect and identify Xoo. The PCR analysis using XOR-F;5'-GCATGACGTCATCG TCCTGT-3' and XOR-R2;5'-CTCGGAGCTA TAT GCCGTGC-3' could serve as an effective tool for identification of and study of the behaviour of Xoo.

UV mutation and induction of pathogenicity minus

Among the 27 pathogenic strains of Xoo, one strain (Xoo-19) showed prominent symptoms in the shortest incubation period and resulted in the longest lesion on KDML105 rice leaves and HR on nonhost and was selected for UV mutagenesis to create the attenuated strains. The surviving cells of Xoo after incubation in the dark from inactivation by UV light for the first and second series were screened, based on their colony morphology. Survival colonies which had altered colony morphology from the wildtype were transferred to NB to allow for normal growth before characterization of the pathogenicity minus. Strain Xoo-19 wildtype, when placed on NA and exposed to UV irradiation for 3 min, produced numerous survivors (10-20% survivors and 50% colonies per dish) that were selected primarily to investigate their growth and swimming motility on NB and 0.4% agar, respectively, as a virulence

minus. Since the movement of pathogenic bacteria on or in the host is important in disease progression, 15 mutants that showed reduction in motility on 0.4% agar medium (Figure 4) were chosen for detached leaf assay. The lack of aggregation observed in the mutant might have been due to a lack of flagella, as the flagella genes have been shown to regulate pili production of Xoo (Lim *et al*., 2008). Lim *et al*. (2008) also reported that the type IV pili of Xoo induced swimming motility and promoted bacterial cell attachment to the host plant surface during colonization.

The defective pathogenicities of Xoo mutants and naturally-occurring avirulent strains were determined by either the detached leaf or inoculated plant assays based on a delay in producing any symptoms and no evident enlargement of disease spread, respectively. The mutant strains (M-690 and M-407) showed no visible symptoms resembling those inoculated with a sterile needle until 14 d post inoculation. The naturally-occurring avirulent strain Xoo-7 showed a localized response which failed to enlarge and had no effect on the leaf segments until

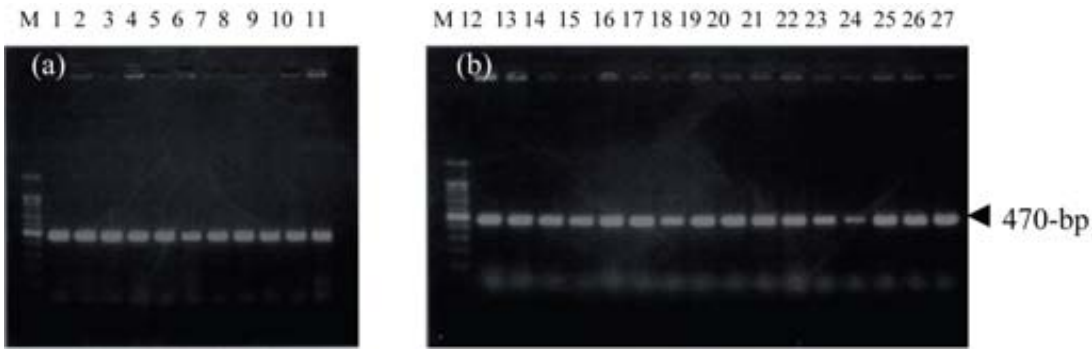

Figure 3 Agarose gel electrophoresis of products from polymerase chain reaction (PCR) performed on DNA of *Xanthomonas oryzae* pv. *oryzae* with 16S-23S rDNA specific primers of XOR-F and XOR-R2. Lane M = 100 bp DNA size markers; lanes 1-27 = amplification at approximately 470-bp of (a) strains Xoo-1 to Xoo-11; and (b) Xoo-12 to Xoo-27.

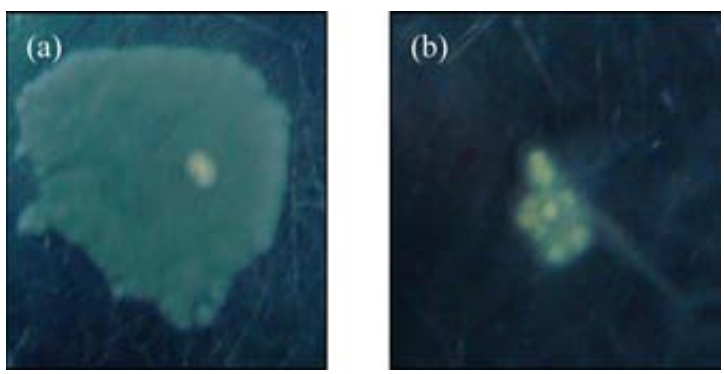

Figure 4 Effect of UV mutation (65 W for 3 min) on Xoo motility. Motility of Xoo-19 wildtype is demonstrated with mass aggregation (a), the UV mutant strain M-690 was no longer motile and unable to produce a pellicle (b).

14 d after inoculation. For the hypersensitive response test, all three strains (Xoo-7, M-690, and M-407) failed to elicit HR on tobacco and tomato leaves, while the wildtype showed clear necrotic lesions within 24 h after infiltration (Figure 1). The wildtype showed water-soaked lesions 6 h after inoculation and 90-95% successful infection was obtained with comparable symptoms to a natural epidemic upon pathogenicity testing of the inoculated, plants. The lesions started with pale-green to grey-green water-soaking near the inoculation point. Then, these lesions coalesced and became yellow at 14 d post inoculation and the whole leaf segments turned yellowish-white later (Figure 2).

All three of M-690, M-407 and Xoo-7 showed lower growth rates when compared with the wildtype. The total population of mutants increased slightly during the first 24 h post inoculation and remained lower than the wildtype throughout the experiment (data not shown). These results suggested that the multiplication of UV-mutants was deficient, resulting in less epiphytic fitness of plant tissue in the pathogenicity phase (Lim *et al*., 2008).

Characterization of non-pathogenic mutant

The stability of the Xoo mutant to pathogenicity was determined with different strategies and subculturing on NA plates over 3 months. After each subculture, the mutant was tested for its ability to produce HR on the nonhost before use.

Bacteriocin production

Mutants from strains M-690 and M-407 and the naturally-occurring avirulent Xoo-7 showed the largest inhibition zones in the paper disc diffusion method against the sensitive pathogenic strain of the wildtype Xoo-15 for bacterioicin production assay compared to the strain Xoo-19 wildtype (Figure 5). Exposure of Xoo to UV irradiation induced a marked increase in RNA polymerase activity and it may have contributed to the transcription of specific genes that occurred in response to the DNA damage (Lin *et al*., 2001). The production of bacteriocins is a common phenomenon by the genus *Xanthomonas* that inhibit the growth of another pathovar (Fett *et al*., 1987). In the present study, UV-irradiated mutant strains (M-690 and M-407) were found to produce larger inhibition halos than those of the parental strains. The greater production of bacteriocins from the mutants in the present study was probably due to UV induction contributing to DNA damage. Parret *et al*. (2003) revealed that the production of bacteriocin from *Pseudomonas* sp. was enhanced by DNA-damaging treatment of producer cells.

Antibiotic marker strains and antagonistic interaction

Tolerance to antibiotics was the criterion used to distinguish mutants from wildtype strains. In the Xoo-antibiotic tests, 25, 50, and 75 μg/mL of rifampicin (Rfm), ampicillin (Amp), and

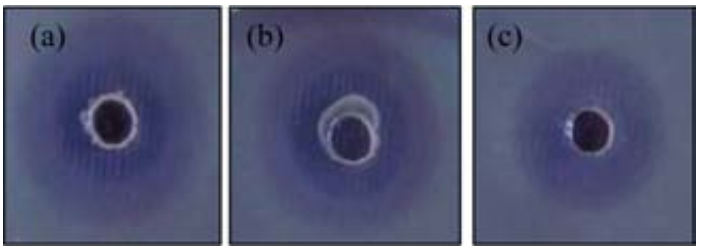

Figure 5 UV mutants of *Xanthomonas oryzae* pv. *oryzae,* M-690 (a), and M-407 (b) produced higher bacteriocin and showed larger inhibition zones against the sensitively pathogenic strain Xoo-15 than did the Xoo-19 wildtype (c).

streptomycin (Stp) were found to be markers of the strains wildtype Xoo-9, mutant M-690 and M-407, respectively. These antibiotic markers were used to monitor the representative strain survival for all experiments. To further characterize the ability of mutants as antagonistic interactions against the wildtype strain, each M-690$^{Amp50\mu g/mL}$ and M-407$^{Stp75\mu g/mL}$ was mixed separately with Xoo-19$^{Rfm25\mu g/mL}$ strains in different ratios and inoculated into the detached leaves, as previously described. A bacterial growth curve was plotted to monitor the accumulation of each strain in mixed inoculation to the leaves by detached leaf assay, so that the Xoo-19 wildtype could not be detected from the mixed inoculation after 12 d (Figure 6a). The results showed that lesion development by Xoo-19 was inhibited at 1:4 of the mutant:wildtype mixed culture with either M-690 or M-407. A ratio of 1:10 or more, increased the lesion length observed (Figure 6b). Barton-Willis *et al*. (1988) demonstrated that mixed inoculation of incompatible (avirulent) together with compatible (virulent) strains resulted in shorter lesion lengths than in the compatible control of BLB. The ratio of mutant and wildtype inoculation of 1:4 also demonstrated control efficacy by the mutant with superior competition toward the wildtype.

Pathogenicity related factors

Several virulence-related phenotypes were also examined. The results from the study indicated that UV-mutation could deteriorate the expression of important virulence factors, including levels of cellulase, protease and extracellular polysaccharide (EPS) (Figure 7). A characteristic of Xoo that was similar to other *Xanthomonas* species with EPS was the ability to form mucoid colonies when cultured on media

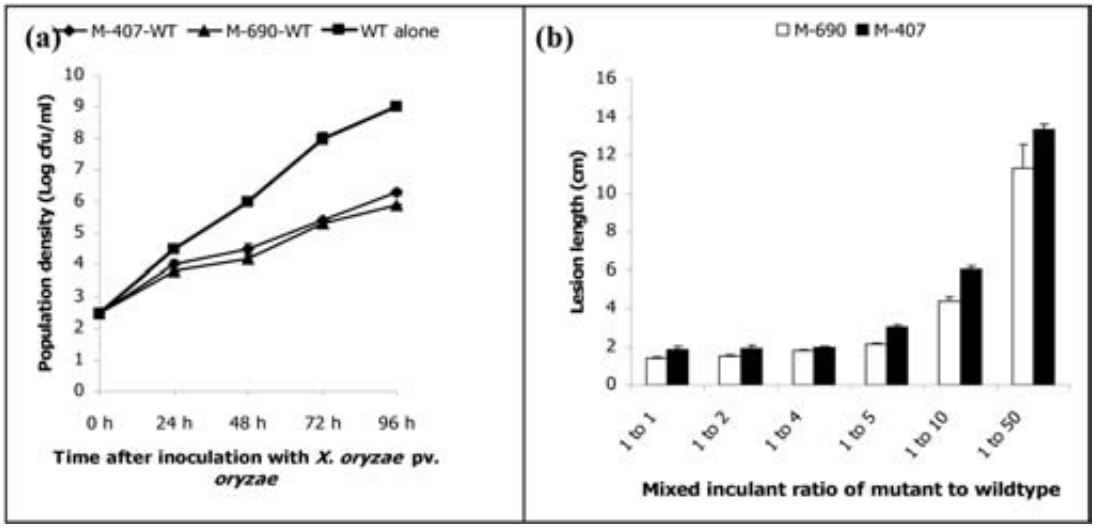

Figure 6 Non-pathogenic UV-mutant strains inhibited the growth of wildtype strain Xoo-19. Growth on KDML105 rice leaves was investigated for strains M-690 and M-407 mixed with Xoo-19 inoculation at different ratios. (a) Inhibition of Xoo-19 by M-690 and M-407 on mixed inoculation by detached leaf assay. (b) Innoculation of KDML105 rice plants at different ratios, (with the ratios, such as 1:1, represented as "1 to 1" etc.). Ratios represent proportion of M-690 (white columns) and M-407 (black columns) with Xoo-19 in the inocula. Columns and the upper limit of the error bars represent mean lesion lengths (measured from 10 leaves) and standard deviation (from three replications), respectively.

supplemented with sucrose (Shen and Ronald, 2002). In this experiment, colonies of mutants were smaller when compared with the wildtype that resulted from less production of EPS. Lee *et al*. (2005) sequenced the whole genome of Xoo and differentiated genetically that EPS could play a critical role in facilitating adhesion of bacteria to the host surface during the initial stages of plant and pathogen interaction and disease development. Also, Ray *et al*. (2000) reported that cellulase, protease and pectate lyase from *Xanthomonas* species played crucial roles in virulence and in bacterial nutrition. The UV mutation showed that less enzyme production was involved in disease development of Xoo in this experiment.

In planta experiment of biological control effectiveness

All treatments of antagonistic bacteria were found to be effective in BLB suppression in this experiment. The ability of M-690, M-407, Xoo-7, and SP007s were effective in reducing BLB severity to 53.7, 49.5, 45.2 and 58.1% in KDML105 and 62.1, 51.4, 47.9 and 62.1% in Suphanburi 1, respectively, when assessed 14 d post inoculation compared to the nontreated control (Table 1). Strain M-690 suppressed disease severity to a level that was not significantly ($P \geq 0.05$) different from the protection pretreated with SP007s, the recommended bacterial antagonist (ISR-P®) as an elicitor of systemic acquired resistance. Rice seedlings inoculated with strain M-407 or naturally-occurring avirulence Xoo-7 had higher disease severity than seedlings inoculated with either SP007s or M-690. The BLB severity was lower and disease suppression was greater in Suphanburi 1 than KDML105 in all coincident inoculations (Table 1).

In the two trials, under disease pressure caused by the Xoo-19 wildtype, the M-690 mutant and SP007s reduced disease severity significantly ($P = 0.05$) and provided similar levels of control (Table 1). The results illustrated the potential of these two strains in suppressing the BLB pathogen.

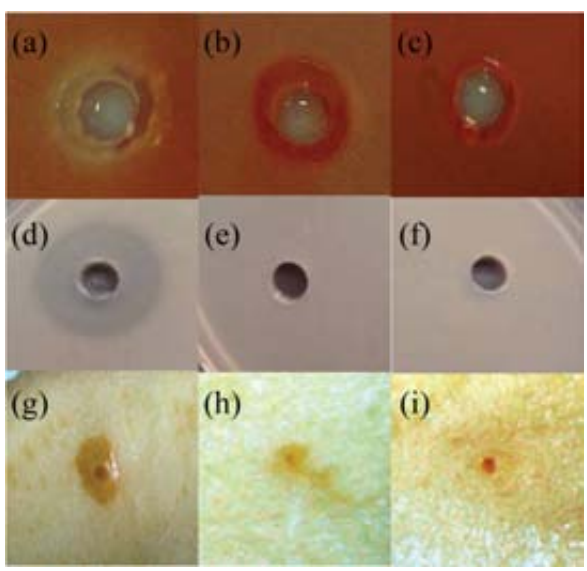

Figure 7 Quantification of exoenzymes produced by the wildtype and mutants of *Xanthomonas oryzae* pv. *oryzae* on medium agar plates. The clear zones around the wells refer to cellulase (a-c), protease (d-f), and pectate lyase (g-i) of the Xoo-19 wildtype, M-407, and M-690, respectively. The last two mutants were defective in these exoenzyme productions.

Under greenhouse experiments, pre-inoculation of the host seedlings by the pathogenic mutant strain M-690 provided protection from the virulent strain Xoo-19. In the control treatments where seedling plants were inoculated solely with a virulent strain, BLB was very severe. This indicated that M-690 was responsible for the reduction in disease severity when it was allowed to precolonize the leaves 3 d before challenge-inoculation with a virulent strain. It was also interesting to note that strain M-690 showed intensive disease reduction to a level statistically equivalent to a strain (*P. fluorescens* SP007s (ISR-P®)) recommended for disease management in rice production (Chuaboon and Prathuangwong, 2008), and provided better control than the naturally-occurring avirulent strain Xoo-7 and the M-407 UV mutant. The antagonistic relationship between avirulent and virulent strains remains unclear, but some possible mechanisms can be explored. Space and resource competition may play a role in the biological control interaction. Over a range of a mixed inoculation, populations of M-690 were consistently higher than those of Xoo-19, although the effective ratio of the mutant was lower than the wildtype in the initial mixed culture. At the infection stage, the ability of Xoo-19 to infect rice seedlings increased as the level of the M-690 mutant in the coincidence was reduced. Biological control of BLB by the Xoo mutant strain might be associated with the ability to colonize rice seedlings. In this case, pre-establishment of the avirulent strain on the leaf surface might consume non-structural carbohydrate and increase multiplication then reduce subsequent colonization by the virulent strain. It was likely that increasing the mutant density on the leaf surface protected the plant from disease by increasing the competition for space and nutrients, thereby decreasing the activity of the pathogen (Dardick *et al.*, 2003). However, in the interactions between the mutant and wildtype co-culture in NB or on the rice leaf surface, increasing the inoculum concentration of the mutant did not result in any further reduction of the disease severity (data not shown). These data indicated that direct competition for infection sites and nutrients was unlikely to be the main mechanism in the reduction of BLB by the M-690

Table 1 Summary of effectiveness of bacterial leaf blight suppression from two replicates having seedling rice cvs KDML105 and Suphanburi 1 co-inoculated with each of the strains SP007s of *Pseudomonas fluorescens* and the mutant and wild type of *Xanthomonas oryzae* pv. *oryzae* under a greenhouse experiment[1/].

Treatment[2/]	Disease incidence (%)[3/]		Disease index (%)[4/]		Disease suppression (%)[5/]	
	KDML105	Suphanburi 1	KDML105	Suphanburi 1	KDML105	Suphanburi 1
ddH2O	66.7^{a}	53.3^{a}	43.3^{a}	40.3^{a}	-	-
M-690	36.7^{b}	34.3^{b}	19.3^{c}	15.3^{c}	53.7^{a}	62.1^{a}
M-407	43.3^{b}	43.3^{a}	20.7^{bc}	19.6^{b}	49.5^{ab}	51.4^{b}
Xoo-7	46.7^{b}	45.0^{a}	23.1^{b}	21.0^{b}	45.2^{b}	47.9^{b}
SP007s	36.7^{b}	34.3^{b}	17.5^{c}	15.3^{c}	58.1^{a}	62.1^{a}

1/ Means within a column followed by the same letter are not significantly different (P = 0.05) according to Fisher's LSD test.

2/ All treatments were co-inoculated with virulent Xoo-19 wildtype.

3/ Diseases severity was assessed 14 d after inoculation with virulent Xoo-19 wildtype. Disease incidence was quantified as percentage of the plants infected.

4/ Disease index was calculated by using the equation DI = 100 x Sum of individual scores / Total leaves observed x Maximum score.

5/ The extent of disease suppression attributed to each treatment was calculated using the equation: Disease suppression efficiency = [(Disease index of control - Disease index of treatment group)] / (Disease index of control) x 100.

mutant; it was probably due to the abundant production of bacteriocin, as mentioned above, and the accumulation of β-1,3-glucanase and peroxidase enzymes induced by the non-pathogenic mutants of Xoo, as shown by one of the experiments hereafter.

Induction of resistance by UV-mutant

Induction of defense mechanism events revealed that the highest accumulation of β-1,3-glucanase was observed in rice leaves treated with SP007s followed by M-690. The accumulation of β-1,3-glucanase increased 1 d after pathogen (Xoo-19 wildtype) inoculation and reached a maximum of 12.7, 12.4, 11.7 and 11.4 µg glucose min^{-1} mg^{-1} protein at the 3rd day with SP007s, M-690, M-407, and Xoo-7 preinoculation, respectively, in cv. KDML105. Even though the activity of β-1,3-glucanase was not significantly different after 3-5 d, all tested strains maintained a higher level compared with the distilled water treatment. In the study, the pre-treatment of the rice plants with tested strains 3 d prior to inoculation with Xoo-19 provided the highest disease reduction rate for both KDML105 (susceptible) and Suphanburi 1(resistant) cultivars. Moreover, the maximum accumulation of β-1,3-glucanase of 15.0, 14.2, 12.8 and 12.8 µg glucose min^{-1} mg^{-1} protein from SP007s, M-690, M-407 and Xoo-7 were observed in the resistant cultivar at the 4th, 4th, 3rd and 3rd d after challenge-inoculation with Xoo-19, respectively (Figures 8a and 8b). Another defense-related enzyme group studied in this experiment was POX, with the maximum activities being observed with 1.5, 1.5, 1.4 and 1.4; and 1.7, 1.6, 1.4 and 1.4 min^{-1} mg^{-1} protein at the 4th, 3rd, 4th and the 4th d after challenge-inoculation with Xoo-19 in rice KDML105 and Suphanburi 1 leaves pretreated with SP007s, M-690, M-407 and Xoo-7, respectively (Figures 8c and 8d). Previous studies have shown that SP007s was an effective biocontrol agent of rice diseases caused by several plant pathogens and investigations have focused on the forms of induced resistance exerted by this biocontrol agent (Chuaboon and Prathuangwong, 2008). It is likely that a non-pathogenic mutant colonized the leaf surface before it could trigger a defense response and this colonization caused M-690 to compete directly with the pathogenic mutant for space and nutrients. The metabolic response characteristic of systemic defense mechanisms, such as the induction of enzymatic activities (POX or β-1,3-glucanase) have been widely described (Sticher *et al*., 1997).

However, there are other metabolic responses triggered by non-pathogenic bacteria that can be appreciated only upon pathogen challenge and consist of a more rapid and stronger defense expression called 'priming' (Conrath *et al*., 2001). Unfortunately, the defense-related enzyme activity was not detected on the 1st day of M-690 preinoculation throughout this study, but other experiments have shown evidence of priming using biocontrol agents (either KPS46 or SP007s pretreatments) and challenge-inoculation with pathogens (Prathuangwong and Buensanteai, 2006; Chuaboon and Prathuangwong, 2008). In the present investigation, all biocontrol strains tested were found to be effective in reducing the incidence of BLB disease in rice plants. However, there is limited information available on plant-mediated defense reactions induced by attenuated mutants of Xoo. Kagale *et al*. (2004) reported that the optimum time of application for strain 13-1; *Lysobacter antibioticus* as a biological control agent of rice bacterial blight was 3 d prior to pathogen inoculation. The same result was obtained by Ji *et al*. (2008), who revealed that the shortest lesion lengths were obtained from plants treated with acibenzolar-S-methyl (ASM), a chemical inducer, when it was added to the rice plants 3 d prior to pathogen inoculation. The results in the present study also agreed with such findings. The study indicated that the activities of PR protein or β-1,3-glucanase and POX were induced by all

tested strains after challenge-inoculation with pathogenic Xoo. PR proteins or β-1,3-glucanase have been well studied with regard to their ability to provide a major defense response in several dicotyledonous plants, both in *R* gene-mediated resistance and in systemic acquired resistance (SAR). Expression of glucanase genes was also activated in rice leaves by infection with blast fungus and treatments with elicitors and chemicals (Song and Goodman, 2001). Moreover, Chittoor *et al.* (1997) revealed that increases in the activity of specific extracellular peroxidases were associated spatially and temporally with a decrease in the rate of pathogen multiplication and spread, suggesting an active role for peroxidases in resistance against bacterial leaf blight. In addition to this, genes for peroxidases were also induced by infection with *Magnaporthe grisea* (McGee *et al.*, 2001). Xoo are found primarily in the vascular tissue or extracellular spaces and they do not penetrate the host cells. However, bacterial multiplication and movement may be inhibited by toxic phenolic compounds, phenolic free radicals and activated oxygen, all of which are associated with lignin-like polymerase accumulated by POX (Reimers *et al.*, 1992). Prathuangwong and Buensanteai (2006) revealed that the plant-pathogen interactions also triggered initial activity in defense enzymes, but later, the activity declined substantially when the pathogen colonized the leaf

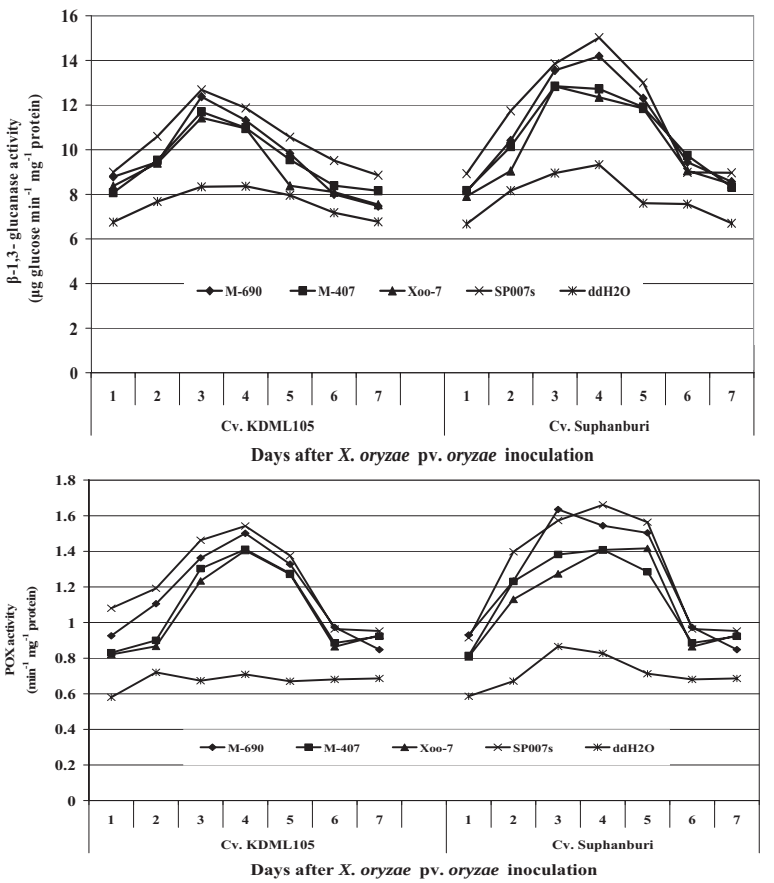

Figure 8 Induction of β-1,3-glucanase (a) and (b) and POX (c) and (d) activities in rice cv. KDML105 ((a) and (c)) and Suphanburi 1 ((b) and (d)) by UV mutants compared with the naturally-occurring avirulent strain (Xoo-7), biocontrol agent (SP007s) and distilled water (ddH_2O).

tissue and the diseased symptoms were visible. In the present study, collectively, the accumulation of β-1,3-glucanase and POX in rice leaves might contribute to induced resistance in rice plants against Xoo.

Like many other plant species, rice employs a diverse array of defenses that minimize the losses during pathogen attack. For example, phenolic compounds toxic to Xoo are found in greater amounts in the healthy leaves of a resistant cultivar compared with the healthy leaves of a susceptible cultivar. In addition to this, increases in POX and β-1,3-glucanase activity are correlated with reduced severity of disease on rice inoculated with the mutant and wildtype of Xoo. In the present greenhouse experiment, a substantial increase in POX activity was observed in extracts from rice leaves aged 1 d after challenge-inoculation with pathogenic Xoo-19. The maximum accumulation was observed on day 4 of post inoculation, which agreed with Reimers *et al*. (1992), who revealed that POX activity decreased in a compatible interaction rather than an incompatible interaction with pathogenic Xoo.

The highest disease suppression was found in the resistant cultivar in all tested strains, although M-690 also had the ability to protect a susceptible cultivar from BLB infection. This result suggested that the biocontrol efficacy of the tested strains was affected by the genetic variability of different cultivars. Ji *et al*. (2008) also found that the biocontrol efficacy of *Lysobacter antibioticus* strain 13-1 on BLB of rice was highly effective on resistant cultivars and concluded that there was some relationship between biocontrol efficacy and the plant cultivar. Perhaps this is a specific interaction between biological control strains and rice cultivars, or perhaps biological control may be most effective on cultivars with some genetic tolerance to BLB.

CONCLUSION

In the characterization of the bacterial pathogen responsible for BLB and the establishment of differences in pathogenicity in rice plants, 27 strains identified as Xoo were isolated from rice leaves showing BLB symptoms in Thailand. The UV-induced mutants differed considerably from the Xoo wildtype in appearance, growth habit and in metabolite and bacterioicin production. Over the period of the experiment, treatment of avirulent M-690 UV mutant reduced BLB severity and provided disease suppression equal to the strain SP007s biocontrol agent (ISR-P®) under greenhouse conditions. The disease suppressive mechanisms of these biocontrols, especially by M-690, included competition for substrate and production of bacteriocin, where induction of systemic resistance was likely the main mechanism. The two UV mutants of Xoo, strains M-690 and M-407, each showed high levels of POX and β-1,3-glucanase activity and inhibition of Xoo pathogen growth, when the host was preinoculated with mutant 3 d before pathogen inoculation that indicated the priming evident. Thus, the mutants obtained, had good potential for use as biocontrol agents for BLB of rice. Additional field trials would be needed to determine the efficacy and stability of these mutant strains. Also, an integrated management approach utilizing resistant or less susceptible cultivars should have excellent potential in a BLB management program.

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