

# Biochemical Approach for Virulence Factors' Identification in *Xanthomonas Oryzae* Pv. *Oryzae*

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## Abstract

Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) leads to a substantial yield reduction of up to 50% in most rice-growing regions. Host plant resistance is an effective control method, and more than 30 resistance genes have been identified in rice genotypes. To understand the interaction of the pathogen in a susceptible reaction of the host plant, *Xoo* culture filtrate and treated culture filtrates were used to treat two rice genotypes using four strains Mai1, PXO88, Dak1 and Dak16. The study revealed that *Xoo* culture filtrate, heated culture filtrate and proteinase K treated culture filtrate induced typical bacterial blight symptoms on rice genotypes IRBB4 and FKR14 with a maximum lesion length of about 23.1 cm for culture filtrate. Heated culture filtrate phytotoxicity effects on both rice genotypes was with highest lesion length of about 6.9 cm, while 13.4 cm was the maximum length induced by a proteinase K treated fraction. After ethyl acetate treatment of the culture filtrate, a considerable reduction of the phytotoxicity was observed. Therefore we suggest that a low molecular-weight toxin may be present in the ethyl acetate extract should not play a major role in *Xoo* virulence and speculate that EPS, Xylanase, polygalacturonase, proteinase contribute to *Xoo* virulence.

**Keywords:** *Xanthomonas oryzae* pv. *Oryzae*; Culture filtrate; Phytotoxicity; Rice; Virulence

## Introduction

*Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes an important rice disease called bacterial blight. Rice bacterial blight was first reported in 1884 in Fukuoka, Prefecture of Japan and is today one of the most important rice diseases in Asia and Africa, implying huge economic consequences. Bacterial blight is the economically most important rice disease in the tropics Mew, Mew et al. [1,2] yield losses associated with the disease are up to 50% of the total yield [3]. During infection *Xoo* produces virulence factors such extracellular polysaccharides (EPS), extracellular enzymes, iron chelating siderophores and effectors of type III secretion [4,5]. These virulence factors were identified using molecular approach. The virulence factors play an important role in successful establishment of *Xoo* in the host plant. EPS such as xanthan and lipopolysaccharides (LPS) produced by *Xanthomonas* genus are involved in disease development [6]. It is also known that a diffusible signal factor (DSF) is required for virulence in *Xoo* [4,7-9].

In pathogenic fungi, a secondary metabolite production is well studied and their role in plant infection and their toxicity to humans are well described. Among plant pathogenic bacteria such as *Xanthomonas* spp. toxin production is reported in *X. albilineans* which produces albicidin, known as virulence factor [10]. A non-ribosomal peptide synthetase-polyketide synthase (NRPS-PKS) enzyme related to syringomycin (SyrE) responsible for syringomycin phytotoxin in *Pseudomonas syringae* is found in *X. axonopodis* pv. *citri*, but its toxins production is not known [11,12].

Erdman et al. [13] reported that Rhizobia strains induce chlorosis on young soybean leaves. The toxic compound was later purified from *Bradyrhizobium elkanii*, and called rhizobitoxine, its phytotoxicity was demonstrated on new soybeans leaves [14]. Rhizobitoxine has been identified as enol-ether amino acid (2-amino-4-[2-amino-3-hydroxypropoxy]-trans-3-butenoic acid), with a molecular weight of 190. Rhizobitoxine has been reported to be an important virulence

factor in many human and animal pathogenic bacteria and is found in many plant pathogenic bacteria such as *X. fastidiosa*, *Rhizobium leguminosarum* and *Erwinia carotovora* [15,16]. In the *Xoo* genome, two apparent RTX toxins, rtxA and rtxC, were identified after genome sequencing of the Korean *Xoo* strain KACC10331[17], but to date the virulence role of the RTX toxins is not yet proved.

Many studies had identified *Xoo* virulence factors using the molecular approach while little is known of their biochemical properties. To better understand the *Xoo* interaction with its host plant, a biochemical approach is needed. Earlier studies revealed that *Xoo* may produce several toxins such as phenylacetic acid (PAA), trans-3-methylthio-acrylic acid (MTAA) and 3-methylthio-propionic acid, which can cause wilting and chlorosis on its host [18]. *Xoo* culture filtrate inhibited rice seed germination [19], but the phytotoxic compounds from *Xoo* and their role in pathogen virulence in the rice-*Xoo*-pathosystem is still not documented. Our study was conducted to identify virulence factors in *Xoo* through phytotoxicity effects of four different treatments of culture filtrate.

## Materials and Methods

To determine the phytotoxicity effects and identify virulence factors, experiments with liquid culture of the bacteria were conducted. Four *Xoo* strains (Mai1, PXO88, Dak1, and Dak16) were selected

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according to their virulence and location of origin, Mai1 from West Africa (African race 3), PXO88 from Philippines (race 9) and Dak1, Dak16 from Tanzania (East Africa). Two rice genotypes (FKR 14 and IRBB4) were used. The rice plants were grown for 3 weeks in the greenhouse and transferred into growth chamber for one week before inoculation under 28°C temperature and 78% relative humidity on the fourth week after transplanting.

The strains were grown on solid modified Wakimoto's medium (0.05 g of Fe<sub>2</sub>SO<sub>4</sub>, 0.82 g of Na<sub>2</sub>PO<sub>4</sub>, 0.5 g of Ca (NO<sub>3</sub>)<sub>2</sub> 4H<sub>2</sub>O, 5 g of peptone, 15 g of agar and 20 g of sucrose dissolved in 1 liter of distilled water) for 48 h and a single colony was used to inoculate 300 ml modified Wakimoto's liquid medium and incubated on a shaker for three days at 28°C and 180 rpm. After incubation, the liquid culture was centrifuged at 4754 x g for 10 min and the supernatant filtrated by passing it through a membrane filter with 0.45 µm pore size. For the following experiments, the culture filtrate was divided into 4 fractions: culture filtrate, boiled culture filtrate, culture filtrate with proteinase K and ethyl acetate extraction.

### Culture filtrate experiment

Four week-old rice plants of rice genotypes IRBB4 and FKR14 were inoculated, by cutting the leaf tips with a pair of scissors priory dipped in the culture filtrate as described by [19,20].

### Boiled culture filtrate

One fraction of the culture filtrate was heated at 80°C for 10 min in water bath to kill the bacteria if still present in the filtrated culture and to inactivate enzymes. The heated culture filtrate was cooled down before use to inoculate rice plants by the leaf clipping method as described above.

### Culture filtrate with addition of proteinase

The third fraction of the filtrated culture was heated at 40°C for 20 min followed by addition of proteinase K (5 µg/ml) and heated at 70°C for 10 min. By addition of proteinase K, protein is degraded in the culture filtrate; the heated and treated mixture was centrifuged at 4754 x g for 10 min and the supernatant transferred into a new tube and used to inoculate rice plants by leaf clipping.

### Ethyl acetate extraction

A culture filtrate of 100 ml volume was extracted with an equal volume of ethyl acetate. The aqueous phase (ethyl acetate phase) was concentrated using a rotary evaporator until 75% of ethyl acetate were evaporated. The 25% left of the aqueous phase were transferred into a 50 ml falcon tube and evaporated using speed vacuum. The product obtained after evaporation was re suspended with 2 ml of 100 mM NaCl and used to treat the rice plant by leaf clipping as described above. The control was performed with non-inoculated modified Wakimoto's medium. The bacteria suspension with 10<sup>6</sup> CFU/ml was used as positive control.

### Evaluation and analyses of phytotoxicity and symptoms on rice genotypes IRBB4 and FKR14

For each experiment, phytotoxicity or disease development were assessed by measuring the phytotoxicity effect on leaves as symptom length and the leaf length. Plants were checked daily and the data from 14 and 21 days post treatment were used to build a graph with mean lesion length induced by each strain on rice genotypes IRBB4 and FKR14. For ethyl acetate extract, the lesion length induced by the

control (medium) was subtracted from the lesion length induced by each strain to receive the effect of the ethyl acetate extract. The whole experiment was replicated three times and the mean values were used for statistical analysis.

Statistical analyses were performed with Statistica software version 7. General Linear Models (GLM) were run for pair wise comparison of culture filtrate treatment to others and for all treatments.

## Results

### Virulence of *Xanthomonas oryzae* pv. *oryzae* strains from different origin on rice genotypes IRBB4 and FKR 14

The lesion length induced on leaves varied from 3.6 cm to 26.7 cm for IRBB4 and from 14.7 cm to 26.7 cm for FKR14, with the maximum lesion length of 26.7 cm at 14 and 21 dpi induced by the Asian strain PXO88. The West African strain Mai1 induced a lesion length of 14.7 cm on genotype FKR14, while the East African strains Dak1 and Dak16 reached medium values with 20 and 21.5 cm of maximum lesion length, respectively, on rice genotype FKR14 (Figure 1).

*X. oryzae* pv. *oryzae* strains Mai1, PXO88, Dak1 and Dak16, selected for the biochemical analyses of virulence factors were virulent on rice genotypes IRBB4 and FKR14. The highest virulence level was exhibited by the Asian strain PXO88. A difference was found on the virulence levels of PXO88 between genotypes IRBB4 and FKR14 at 14 dpi, and at 21 dpi, the virulence level was nearly the same on both genotypes with 26.7 cm of lesion length. The West African strain Mai1 did not show a high virulence level at 14 dpi and revealed to be the least virulent strain. All the strains showed a virulence progression on the rice genotypes except strain PXO88 which was highly virulent already after 14 dpi, infecting the whole leaf length of FKR14 after 14 dpi. The East African strains Dak1 and Dak16 were more virulent than the West African strain (Mai1), (Figure 1).

### Phytotoxicity of culture filtrate

*X. oryzae* pv. *oryzae* culture filtrate of strains Mai1, PXO88, Dak1

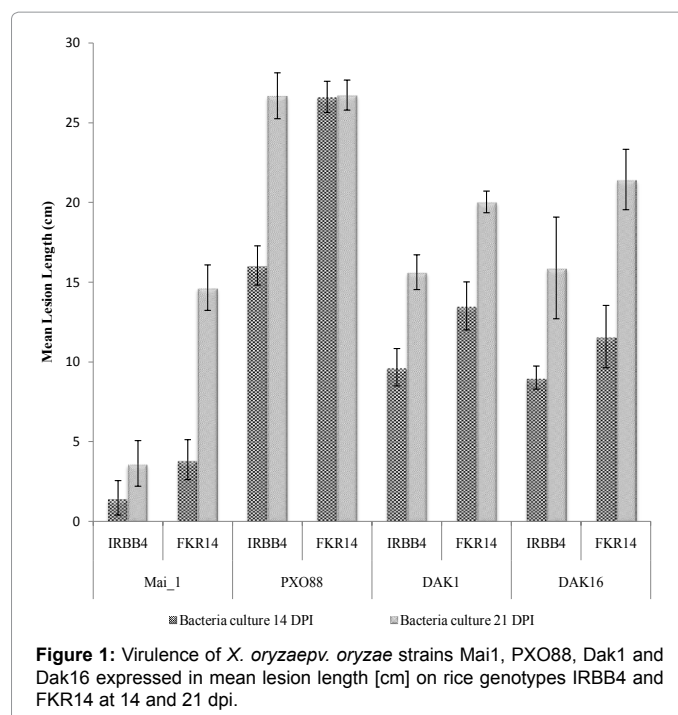
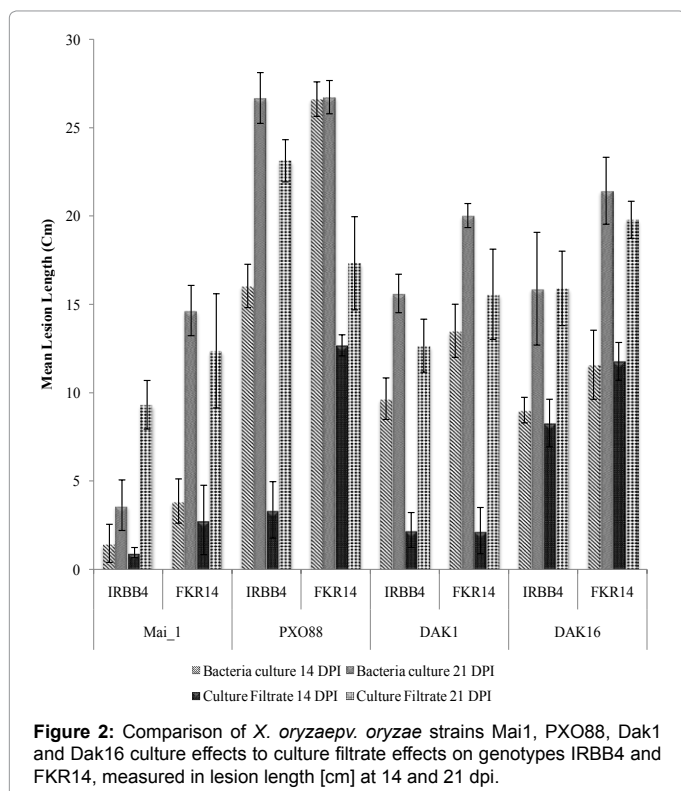


Figure 1: Virulence of *X. oryzae* pv. *oryzae* strains Mai1, PXO88, Dak1 and Dak16 expressed in mean lesion length [cm] on rice genotypes IRBB4 and FKR14 at 14 and 21 dpi.



and Dak16 induced phytotoxicity effects 14 and 21 days after leaf clipping treatment on both rice genotypes IRBB4 and FKR14. The phytotoxicity effects of *Xoo* culture filtrates on both genotypes in comparison to bacteria culture effects are presented as lesion length on leaves (Figure 2). Generally, lesion lengths at 14 dpi were lower in treatment with culture filtrate than in treatments with bacteria culture, with exception of strain Dak16, where lesion lengths from culture filtrate were similar to those from bacteria culture already at this time point. At 14 dpi the phytotoxicity effect of culture filtrate varied from 0.95 cm to 12.7 cm lesion length with the highest lesion length induced by strain PXO88 on FKR14. The phytotoxicity effects scored at 21 dpi showed a variation from 9.3 cm to 23.1 cm induced by strains Mai1 and PXO88, respectively, on rice genotype IRBB4. At 21 dpi lesions induced by culture filtrate had reached higher values and were comparable to the lesion induced by bacterial cultures in treatments with strain Dak16 on both rice genotypes, while the other strains values from culture filtrate treatment remained lower than the value from bacterial culture, except on the interaction between strain Mai1 and rice genotype IRBB4.

The statistical analysis of data derived from the 21 dpi measurement revealed significant differences between bacterial culture and culture filtrate treatments, strains and rice genotypes: bacteria culture induced higher lesion lengths than culture filtrate, strain PXO88 demonstrated higher effect on rice genotypes than the other strains, while rice genotype FKR14 was more susceptible than genotype IRBB4 (Table 1). Significant differences were also observed within treatments and strains, with culture filtrate of strain PXO88 inducing lower lesion length than its bacteria culture, and between treatments and rice genotype, with generally higher lesion lengths induced by culture filtrate on rice genotype FKR14 than on genotype IRBB4. The interaction of treatments, strains and rice genotypes was not significant.

### Phytotoxicity of heated culture filtrate

The phytotoxicity effect of heated culture filtrates of *Xoo* strains Mai1, PXO88, Dak1 and Dak16 inoculated to rice genotypes IRBB4 and FKR14 was first observed one week after treatment. Lesions induced by heated culture filtrate were generally lower at 14 dpi and in all treatments lower at 21 dpi than values from non-heated culture filtrate (Figure 6). Lesion length from heated culture filtrate generally increased from 14 to 21 dpi (Figure 3). The lesion length induced by heated culture filtrate varied from 1.8 cm to 5.1 cm with rice genotype IRBB4 and from 3.3 cm to 6.9 cm on FKR14 at 21 dpi (Figure 6).

Figure 6 and statistical results (Table 2) show significant differences between culture filtrate and heated culture filtrate treatments, with higher phytotoxicity effect of culture filtrate compared to heated culture filtrate on both rice genotypes. Significant differences were also observed between strains and rice genotypes, with strains PXO88 and Dak1 inducing the highest phytotoxicity effect on both rice genotypes, while strain Mai1 showed a lower lesion length on rice genotype IRBB4. Significant interactions with p-value 0.00 were also recorded between treatments and strains, with strain PXO88 culture filtrate showing a higher divergence on lesion length compared to heated culture filtrate on rice genotype IRBB4 than on genotype FKR14.

### Phytotoxicity of culture filtrate treated with proteinase K

Inoculation of culture filtrate of strains Mai1, PXO88, Dak1 and Dak16 treated with proteinase K to rice genotypes IRBB4 and FKR14 lead to a significant reduction in phytotoxicity lesion lengths compared to inoculation with untreated culture filtrate (Figure 6). Differences were higher at 21 dpi than at 14 dpi. Only in the interaction strain

	SS	DF	MS	F	P
Treatments	64.98	1	64.98	18.134	0.000169***
Strains	1122.08	3	374.03	104.372	0.000000***
Rice Genotypes	116.53	1	116.53	32.518	0.000003***
Treatments x Strains	112.22	3	37.41	10.438	0.000061***
Treatments x Rice Genotypes	53.57	1	53.57	14.949	0.000509***
Strains x Rice Genotypes	161.94	3	53.98	15.063	0.000003***
Treatments x Strains x Rice Genotypes	22.55	3	7.52	2.097	0.120142
Error	114.68	32	3.58		

Note:\*\*\*p-value highly significant at 0.01; SC: Sum of Square; DF: Degree of freedom, MS: Mean square; F: F of Fisher; p: probability

**Table 1:** Statistics of comparison of *Xanthomonas oryzae* pv. *oryzae* culture effects to culture filtrate effects at 21 dpi.

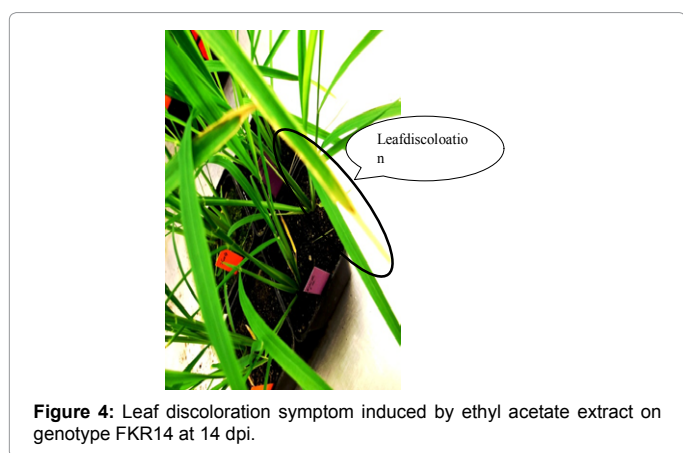




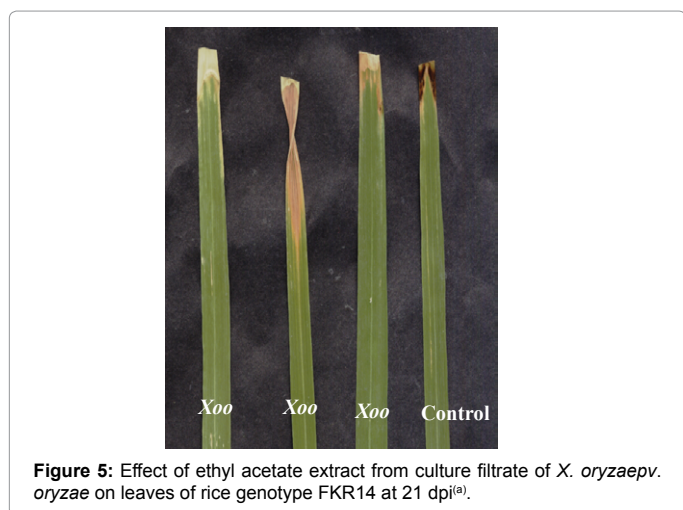
	SS	DF	MS	F	P
Treatments	1467.330	1	1467.330	306.030	0.000000***
Strains	170.705	3	56.902	11.868	0.000022***
Rice Genotypes	36.453	1	36.453	7.603	0.009548***
Treatments x Strains	164.669	3	54.890	11.448	0.000029***
Treatments x Rice Genotypes	6.564	1	6.564	1.369	0.250631
Strains x Rice Genotypes	46.760	3	15.587	3.251	0.034465**
Treatments x Strains x Rice Genotypes	63.189	3	21.063	4.393	0.010683**
Error	153.431	32	4.795		

Note:\*\*\*p-value highly significant at 0.01; \*\*p-value significant at 0.05; SC: Sum of Square; DF: Degree of freedom, MS: Mean square; F: F of Fisher; p: probability

**Table 2:** Statistics of comparison of *Xanthomonas oryzae* pv. *oryzae* culture filtrate effects to heated culture filtrate effects at 21 dpi.



**Figure 4:** Leaf discoloration symptom induced by ethyl acetate extract on genotype FKR14 at 14 dpi.



**Figure 5:** Effect of ethyl acetate extract from culture filtrate of *X. oryzae* pv. *oryzae* on leaves of rice genotype FKR14 at 21 dpi<sup>(a)</sup>.

Dak1 on rice genotype FKR14, values from untreated and proteinase K treated culture filtrate were similar. At 14 dpi, inoculation with culture filtrate treated with proteinase K resulted in minimum and maximum lesion lengths of 0.6 cm and 1.7 cm induced by strain Dak1 on rice genotypes IRBB4 and FKR14, respectively. The phytotoxicity effect of proteinase K treated culture filtrate increased at 21 dpi, varying on rice genotype IRBB4 from 1.5 cm to 5.4 cm and on rice genotype FKR14 from 4.1 cm to 13.4 cm. at 21 dpi, with maximum lesion lengths of 5.4 cm, 5.9 cm, 13.4 cm and 4.2 cm, induced by treated culture filtrates of *Xoo* strains Mai1, PXO88, Dak1 and Dak16 respectively.

The comparison between culture filtrate effects to culture filtrate

treated with proteinase K effects (Figure 5) and the statistical analysis at 21 dpi (Table 3) revealed significant differences ( $p=0.00$ ) between culture filtrate and proteinase K treated culture filtrate, with a lower phytotoxicity effect induced by proteinase K treated culture filtrate than with untreated culture filtrate, while the strain Dak1 induced similar lesion lengths with both treatments on rice genotype FKR14 at 21 dpi.

Significant differences were observed between treatments and rice genotypes ( $p=0.03$ ), where both treatments induced higher phytotoxicity effects on rice genotype FKR14 than genotype IRBB4 at 21 dpi. Only strain PXO88 culture filtrate induced higher lesion lengths on genotype IRBB4 than on genotype FKR14.

### Phytotoxicity of ethyl acetate extract from culture filtrate

*X. oryzae* pv. *oryzae* ethyl acetate fraction from culture filtrate was used to investigate a possible role of small molecules as virulence factors of *Xoo*. Inoculation of culture filtrate extracted with ethyl acetate by leaf clipping resulted in a highly to totally reduced phytotoxicity induction (Figure 6). Nevertheless the phytotoxicity effects of the ethyl acetate fraction after leaf clipping treatment varied from 0.6 cm to 3.4 cm and 1.9 cm to 3.99 cm on rice genotype IRBB4 and on FKR14, respectively, at 21 dpi. The phytotoxicity effects were first observed 3 days after treatment with leaf discoloration symptoms (Figures 4 and 5). At 14 dpi the maximum lesion length of 2.03 cm was recorded, induced by strain Dak1 on rice genotype FKR14. At 21 dpi the maximum mean lesion lengths 3.4 cm and 3.9 cm were induced by Dak1 on genotypes IRBB4 and FKR14, respectively. All the strains demonstrated increases of phytotoxicity effects on both rice genotypes comparing 14 and 21 dpi.

The comparison of phytotoxicity of the ethyl acetate fraction from culture filtrate to non-extracted culture filtrate at 21 dpi revealed

	SS	DF	MS	F	P
Treatments	1298.960	1	1298.960	420.467	0.000000***
Strains	157.310	3	52.437	16.974	0.000001***
Rice Genotypes	52.585	1	52.585	17.021	0.000246***
Treatments x Strains	251.885	3	83.962	27.178	0.000000***
Treatments x Rice Genotypes	14.257	1	14.257	4.615	0.039366**
Strains x Rice Genotypes	121.773	3	40.591	13.139	0.000009***
Treatments x Strains x Rice Genotypes	74.693	3	24.898	8.059	0.000386***
Error	98.859	32	3.089		

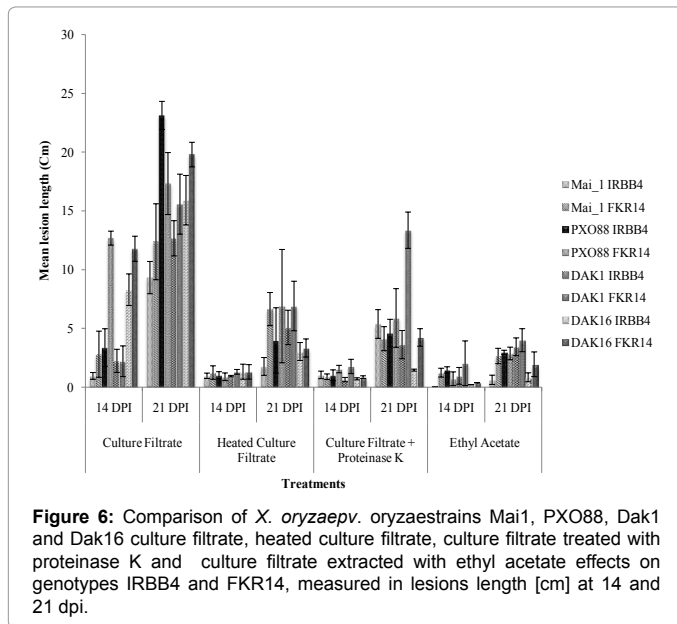
Note:\*\*\*p-value highly significant at 0.01; \*\*p-value significant at 0.05; SC: Sum of Square; DF: Degree of freedom, MS: Mean square; F: F of Fisher; p: probability

**Table 3:** Statistics of comparison of *Xanthomonas oryzae* pv. *oryzae* culture filtrate effects to culture filtrate treated with proteinase K at 21 dpi.

	SS	DF	MS	F	P
Treatment	1724.74	1	1724.74	555.366	0.000000***
Strains	139.209	3	46.403	14.942	0.000011***
Rice Genotypes	9.825	1	9.825	3.164	0.087962
Treatment x Strains	125.881	3	41.96	13.511	0.000023***
Treatment x Rice Genotypes	0.001	1	0.001	0	0.988431
Strains x Rice Genotypes	51.367	3	17.122	5.513	0.005024***
Treatment x Strains x Rice Genotypes	26.518	3	8.839	2.846	0.058835
Error	74.534	24	3.106		

Note: \*\*\* p-value highly significant at 0.01; SC: Sum of Square; DF: Degree of freedom, MS: Mean square; F: F of Fisher; p: probability

**Table 4:** Comparison of effects of *Xanthomonas oryzae* pv. *oryzae* culture filtrate to culture filtrate extracted with ethyl acetate at 21 dpi.



significantly lower lesion lengths induced by ethyl acetate extract compared to culture filtrate effects (Table 4). Significant differences were found between strains, and also the interaction between strains and rice genotypes was significant ( $p=0.005$ ). Treatments and rice genotypes interaction revealed not significant at 21 dpi.

## Discussion

### Strain virulence

The virulence analysis result showed that Asian strain PXO88 is highly virulent compared to African strains used in this study. Also, within African strains, phenotypic differences were found. It has been reported that African *Xoo* strains are genetically distant from Asian strains [21]. Further studies with a higher number of strains are recommended. Two groups have been reported within the West African *Xoo* population [22]. The variability in the *Xoo* population is a consequence of the development of resistance durability of the bacterial races in time and space [23-25].

Near isogenic lines (NILs) with specific resistance genes have been identified and among them, lines with a single resistance gene are widely used to characterize virulence and differentiate pathogenic races of *Xoo*. Classification of the pathogenic race of *Xoo* is based on the interaction between its *avr* gene and rice *Xa* gene [23,26-28]. In this study, we used IRBB4 line carrying *Xa4* gene and the susceptible genotype FKR14. The phenotypic reactions of the bacteria strains and rice line IRBB4 were similar to the reaction observed on FKR14 except with strain Mai1. IRBB4 line shows susceptibility to *Xoo* strains from Iran [28], while in China; it is susceptible to 50% of Chinese *Xoo* strains [29]. In earlier 1970, the *Xoo* populations were dominated by those strains that could not infect rice lines with *Xa4* gene [30], while the newly developed rice lines with *Xa4* gene are nowadays susceptible to several strains [31]. Strain Mai1 revealed non-virulent on IRBB4, confirming previous study on African *Xoo* strains, which reported that African *Xoo* race 3 is not virulent on IRBB4 [21].

### Phytotoxicity effects from *Xoo* culture filtrate and treated culture filtrate on rice

*X. oryzae* pv. *oryzae* strains (Mai1, PXO88, Dak1 and Dak16) culture

filtrate, heated culture filtrate, culture filtrate treated with proteinase K and ethyl acetate extract were used to study their phytotoxicity effect on rice genotypes IRBB4 and FKR14. Culture filtrate had the highest phytotoxicity effect against rice genotypes compared to heated culture filtrate, culture filtrate treated with proteinase K and ethyl acetate extract. The least square mean (LSM) lesion length evoked by bacteria culture was 18.1 cm, while with culture filtrate, heated culture filtrate, culture filtrate treated with proteinase K and ethyl acetate extract the lesion lengths LSM were 15.8, 4.7, 5.4 and 2.4 cm, respectively (data not shown). This result revealed, that the phytotoxicity of culture filtrate is similar to the phytotoxicity produced by bacteria, where strain Dak16 demonstrated no significant difference between bacteria culture lesion (15.9 cm and 21.4 cm) and its culture filtrate phytotoxicity lesion (15.9 cm and 19.8 cm) on rice genotypes IRBB4 and FKR14, respectively. The bacteria cultures showed variations in their reactions on rice genotypes, and the same variations were also found by inoculation with culture filtrate, revealing that phytotoxicity on rice is possibly produced by *Xoo* virulence factors. Significant differences were found between bacteria culture and culture filtrate effects, with higher effects of bacteria culture than culture filtrate with strains Dak16, strains PXO88 and Dak140, with phytotoxicity of culture filtrate (9.3 cm) being higher than bacteria effect (3.6 cm) of strain Mai1 on rice genotype IRBB4.

While the bacteria culture filtrate induced a phytotoxicity effect with a maximum lesion length of up to 23 cm, the heated culture filtrate induced 6.9 cm of mean length. Thus, the heating treatment caused a reduction of the phytotoxicity of the culture filtrate. This results, possibly indicating that the culture filtrate effect was related to lipopolysaccharides, xylanase, polygalacturonase that *Xoo* produced and are reported as typical bacteria virulence factors [6,32] - and which were inactivated or killed by heating. Generally, enzymes are inactivated at higher temperature. The optimum temperature for the activity of *Bacillus subtilis* xylanase is 55°C, and 0-45°C for *Aspergillus flavus* polygalacturonase, and while at 80°C 25% activities remained [33,34]. Therefore, the low phytotoxicity observed with heated culture filtrate in this study with 80°C of heating temperature should have affected the enzymes' activity in the culture filtrate.

The culture filtrate treated with proteinase K revealed generally lower phytotoxicity effect compared to other treatments, though the lesions were slightly larger than with heated culture filtrate. This finding supported the hypothesis that *Xoo* culture filtrate phytotoxicity is due to enzymes' activities or to proteins. Higher effect of culture filtrate treated with proteinase K could be due to presence of proteinaceous in the culture filtrate which perhaps inhibits the activity of related enzymes. Proteins including adhesion proteins are secreted via the Two Partner Secretion System (TPS) and contribute to virulence by bacteria attachment to the host surface [7,35]. *Xoo* produces proteins that are required for virulence, and the inactivation of proteins in heat-treated *Xoo* culture filtrate would be related to the low observed phytotoxicity effect on the rice genotypes. Chatterjee and Sonti [36] reported that *Xoo* protein phytase A (*PhytA*) is required for virulence. *Xoo* strain Dak1 showed 13.3 cm lesion length with culture filtrate treated with proteinase K on rice genotype FKR14, while other strains showed less effect. This result is in contrast to the fact that *Xoo* secreted proteins are required for virulence. Plant-pathogen interactions are related to the plant defense response from the initial recognition event to defense or susceptibility reactions. Generally, plant elicitors in response to a stress can be race specific. This could explain the fact, that *Xoo* strain Dak1 culture filtrate treated with proteinase K overcame the rice genotype FKR14 defense barriers in absence of proteins and induced a similar phytotoxicity effect compared to culture filtrate.

The ethyl acetate extract showed the lowest effect on rice genotypes compared to other treatment revealing that the ethyl acetate extract did not contain an active virulence factor inducing phytotoxicity. Therefore, we suggest, that *Xoo* does not produce low molecular-weight secondary metabolites (toxins) significantly involved in the bacterial virulence against the rice plant. The only phytotoxicity effect observed on rice leaves after treatment was leaf discoloration. Previous study (Shao and Wang, 1997) on *Xoo* culture filtrate revealed that *Xoo* culture filtrate contains some elicitors, which induced a toxicity effect on rice, and induced rapid necrosis on tobacco leaves. Our result also corroborates the data from Kong et al. [18] who found after extracting from bacteria culture with ethyl acetate, that *Xoo* toxins induced leaf discoloration and cell death on rice. A necrotic lesion as observed in our study has been reported from many bacteria species producing phytotoxic compounds. A phytotoxic compound of *Pseudomonas syringae* pv. *sesame* induced necrotic symptoms [37], which were similar to the phytotoxicity of mangotoxin from *P. syringae* pv. *syringae* strain UMAF0158 on mango tree [38]. Several toxins similar to lipodepsipeptide produced by strains of *P. syringae* pv. *syringae* were reported [39-41] such as syringomycins and syringopeptins which induced necrotic symptoms on plants [42,43]. Ethyl acetate extract treatments induce also chlorosis and necrosis on both rice genotypes IRBB4 and FKR14. The phytotoxin coronatine and antimetabolite toxins induced chlorosis and an increase in disease symptoms, respectively, and are considered as virulence factors in *P. syringae* [44-47], while *Xoo* ethyl acetate extract, did not increase disease symptoms, and therefore it is not, that the ethyl acetate extract contains virulence factor with phytotoxic activity.

Differences were seen between treatments, strains and rice genotypes. The culture filtrate effect was highest, and a considerable decrease of phytotoxicity was observed with the other treatments. We do not rule out that *Xoo* might produce a compound to induce phytotoxicity on rice leaves, but this compound may only to a minor extent contribute to the bacterial virulence. On the contrary, Noda et al. [48] identified seven toxic substances in ethyl acetate soluble acidic material: 3-methylthiopropionic acid, *trans*-3-methylthioacrylic acid, phenylacetic acid, isovaleric acid, tiglic acid, succinic acid and fumaric acid, isolated from *Xoo* culture suspension. His culture suspension induced necrosis and chlorosis on rice leaves at higher concentration (2000 µg/ml). We suppose that the contradictory results are due to the fact that Noda et al. [48] extracted the toxic substances from culture suspensions which contain bacteria, but we extracted from culture filtrate where bacteria were removed. Thus, when we also treated bacteria culture suspension with heat and proteinase K instead of treating the culture filtrate as in our general trials, we also found a higher phytotoxicity effect than with culture filtrate treated in the same way. Therefore, we believe that the *Xoo* virulence factors remain in association with the bacteria. Furthermore, secreted proteins including adhesins have been reported to be involved in the bacterial virulence, as well as the diffusible factor signal (DFS) synthase, which belongs to the enoyl-co-A-hydratase family and plays an important role in attachment, biofilm formation and virulence of *Xoo* [49].

The difference between strains could be related to the strains' virulence and/or to the genetic or geographic distance between strains. Gonzalez et al. [21] found that African strains are genetically distant from Asian strains, while Onasanya et al. [22] found two groups within West African *Xoo*. The differences observed between rice genotypes which showed highest effect against rice genotype FKR14 than IRBB4 corroborate the study of Shao and Wang [19]. The biochemical

approach for virulence factors identification in *Xoo* revealed no small metabolites playing a major role in *Xoo* virulence.

## Conclusion

Biochemical assays for virulence factor identification in cultures, culture filtrate, heated culture filtrate, culture filtrate treated with proteinase K and in the ethyl acetate extracted fraction of *Xoo* strains Mai1, PXO88, Dak1 and Dak16 were carried out on rice genotypes IRBB4 and FKR14. Culture filtrate induced phytotoxicity effects on both rice genotypes with lesion lengths comparable to lesion lengths induced by the bacteria culture. A considerable reduction in phytotoxicity effects was observed with proteinase K treated culture filtrate, after heating culture filtrate and with ethyl acetate extract, in reducing order. First symptoms were visible after inoculation of *Xoo* cultures and ethyl acetate fraction at 3 dpi, with culture filtrate at one week and with heated culture filtrate and culture filtrate treated with proteinase K at 10 dpi. Statistical analyses confirmed that the phytotoxicity effects were significantly influenced by strains and by rice genotypes, and also the interactions treatments strains, strains rice genotypes and treatments strains rice genotypes were significant. We suggest, additionally to LPS, polygalacturonase and Xylanase, *Xoo* produces a proteinaceous virulence factor. Further factors possibly attached to the bacterial cells were not the target of the present study.

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