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Pathogenicity Assay and Molecular Identification of Fungi and Bacteria Associated with Diseases of Tomato in Malaysia

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Abstract

This study was conducted in order to determine the fungi and bacteria associated with tomato plants at Cameron Highlands Malaysia. The fungi which have been isolated and detected from tomato plants were: *Fusarium oxysporum*, *F. solani*, *F. acuminatum*, *Rhizoctonia solani*, *Colletotrichum boninense*, *C. acutatum* and *Phoma destructiva*. The bacteria which have been isolated and detected from tomato plants were: *Ralstonia solanacearum*, *Xanthomonas vesicatoria*, *X. gardneri* and *Pseudomonas syringae.* While the most pathogenic fungi were *C. boninense*, *P. destructive* and *F. oxysporum* with the disease incidence (89.6%, 86.6%, 85.6%) respectively, the most pathogenic bacteria were *X. vesicatoria* and *R. solanacearum* with the disease incidence (96.6% and 87.6%) respectively.

Keywords

***Lycopersicon esculentum*, Pathogen Isolation and Molecular Identification**

1. Introduction

An estimated 124.5 million tonnes of tomatoes (*Lycopersicon esculentum* Mill.) are produced annually through-

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out the world, making one of the ten most important fruits and vegetables in modern history [1]. Production of tomatoes tends to be more successful in highland areas in Malaysia, mainly due to the milder temperatures there. Hence, Cameron Highlands is a popular location for large-scale tomato cultivation. There are still a large number of fungal and bacterial diseases that make tomato production challenging in various parts of the world despite decades of conventional breeding and selection. Presently, tomato is vulnerable to more than 200 diseases. Yield losses to diseases can run as high as 70% to 95%. As extensive cultivation of tomatoes continues, diseases caused by bacteria, soil borne fungi, seed borne and foliar fungi have also increased even continuous improvement in strain variety [2].

In greenhouse and open field production, bacterial diseases are a serious problem. In the warm and temperate regions of the world, five major bacterial pathogens are responsible for damages to the roots, stems, twigs, leaflets, leaves, buds, flowers and fruits. These include *Pseudomonas syringae* pv. tomato, the causal agent of bacterial speck, *Xanthomonas vesicatoria*, the causal agent of bacterial spot, *Clavibacter michiganensis* subsp., *Michiganensis*, the causal agent of bacterial canker, *Pseudomonas corrugate*, the causal agent of bacterial pith necrosis and *Ralstonia solanacearum*, the causal agent of bacterial wilt [3]. In Malaysia, economic crops and vegetables such as tomatoes, chillies and groundnuts bacterial are plagued by wilt, a serious bacterial disease, caused by *R. solanacearum* [4] [5].

The most severe fungal diseases that threaten tomato yields and cause worldwide economic losses are the wilts caused by *Fusarium oxysporum* f. sp. *lycopersici*, root rot caused by *Fusarium solani*, damping off caused by *Rhizoctonia solani* and root rot and damping-off caused by several *Pythium* species [6]. The most economically damaging phytopathogenic fungi are *Colletotrichum* sp., which causes anthracnose, and *Phoma destructiva*, which causes *Phoma* rot [7].

Bacterial wilt (*R. solanacearum*), bacterial spot (*X. vesicatoria*), early blight (*Alternaria solani*), anthracnose (*Colletotrichum* spp.), *Sclerotium wilt* (*Sclerotium rolfsii*), damping off (*R. solani*), tomato wilt (*F. oxysporum*) and viruses such as tomato mosaic virus have been reported on tomato in Malaysia [5] [8].

The present study was initiated to isolate and identify tomato pathogens in Cameron Highlands, Malaysia via molecular identification and evaluate the pathogenicity and virulence variability of pathogenic isolates.

2. Materials and Methods

2.1. Pathogens Isolation

Leaf, stem, root and fruit samples of tomato plants with disease symptoms were gathered from various tomato fields in Cameron Highlands, Pahang, Malaysia. Five mm^2 segments were taken from the edge of the different samples in order to isolate the causative fungal and bacterial pathogens. One% NaOCl was then used to surface sterilize the specimens for two minutes. After sterilization, sterilized distilled water was used to rinse the samples twice and then dried on sterilized filter papers.

For fungi isolation specimens were placed into petri dishes with Potato Dextrose Agar (PDA) and kept in an incubator at 28˚C for 3 to 6 days to let the fungi grow. After that, the samples were monitored under a stereomicroscope and conidia or single hyphae were moved to PDA plates with a needle and kept in an incubator until the fungi developed. The obtained pure cultures were used in this study.

For bacteria isolation specimens were moved into small test tubes and soaked in 2 mL of sterilized distilled water for an hour. The tubes were stirred in a vortex mixer to obtain turbid bacterial suspensions. Loopfuls of the resulting aqueous suspension were streaked onto Nutrient Agar (NA) and incubated at 30˚C for 2 days. Different colonies were again streaked onto NA plates and this process was repeated until purified bacterial cultures were obtained with homogeneity colony morphology.

2.2. DNA Extraction and Sequence Analyses

2.2.1. DNA Extraction and Molecular Identification of Fungal Isolates

Fungal mycelial mats were put into 1.5 mL micro tubes and centrifuged at 3000 rpm for 5 minutes at 4˚C. The supernatants were then carefully removed. DNA extraction was performed with the 3% SDS method [9].

The ITS (including, ITS1 and ITS2, 5.8S rDNA) region was selected to characterise isolates using universal primers [10].

2.2.2. DNA Extraction and Molecular Identification of Bacterial Isolates

For 24 hours at 30˚C, each bacteria isolate was cultivated in a nutrient broth. A commercial genomic DNA purification kit was used to extract the total genomic DNA from all the isolates. The 8F forward primer (-5-GTG ACACGTACACGT-3-) and 1492R reverse primer (-5-ATCGCACGTACACGT-3-) were used to amplify he 16S RNA gene. A 25 µl reaction mixture containing 10 ng of genomic DNA, 2.5 µl of 10X PCR buffer, 2.5 µl of $MgCl_2$, 200 µM of dNTP mix, 10 p mol each of the primers and 1 unit of Taq DNA polymerase was used for the PCR reaction. The initial denaturation temperature was set at 95˚C for 5 minutes, followed by 30 cycles of denaturation for 1 minute, annealing at 55˚C for 1 minute and extension at 72˚C for 2 minutes. A final extension was carried out at 72˚C for 5 minutes [11].

2.3. Pathogenicity Assays

For bacteria inoculation preparation, the bacteria were streaked on the NA medium and incubated for 48 hours at 28˚C. NA plate cultures in sterile distilled water were prepared by suspending the growth from 48 hours. Tween 20, as a surfactant, was combined with bacteria suspensions containing about 10^8 cfu/ml and for a final concentration of 0.02% (v/v).

For fungi inoculation preparation, conidial suspension was readied by sampling conidia from seven days old fungal culture isolates grown on PDA using 5 - 10 mL sterilized distilled water. A sterile spatula was used to scrub the surface of the fungus plates. A haemocytometer was used to adjust the concentration of the conidia suspension to 3×10^5 conidia/mL and tween 20 was added as a surfactant for a final concentration of 0.02% (v/v).

The tomato seeds (cultivar Baccarat 322) were disinfected with 50% Benomyl fungicide and streptomycin for 2 hours prior to sowing. Disinfected seeds were hand sown in 231-hole plastic plug trays and placed in the glasshouse under natural daylight. They were also watered daily. Two seedlings were taken from the plug trays and moved to pots, four replications for each treatment.

Four-week old tomato plants were sprayed with a conidia suspension containing 3×10^5 conidia/mL (15 mL/pot) and the bacterial inoculum (10^8 cfu/ml) in distilled water. To increase infection, roots of test plants were wounded with a sharp scalpel by stabbing the soil several times and then pouring of 15 ml of bacterial inoculum and conidia suspension onto surface of the soil. Plants that were not inoculated were sprayed with distilled water that had similar concentration of tween 20. The plants were covered with black plastic bags for 24 hours after inoculation. The plastic bags were removed after 24 h and plants watered daily. Disease incidence and severity [12] were measured seven days after inoculation.

Small sections (1 × 1 cm) of diseased leaves, stems or roots were surface sterilized with 10% of sodium hypochlorite for 1 - 2 minutes and rinsed three times with sterile distilled water for fungus and bacteria isolation,. The segments were then cultured on PDA or NA culture mediums. To prove that Koch's postulates were true, the final colony and conidia characteristics were analysed to confirm that the isolated fungus or bacteria were the same with what had been used for the pathogenicity test.

2.4. Statistical Analyses

SAS software version 9.2 was used to perform the Analysis of Variance (ANOVA) (Cary, NC: SAS Institute Inc. 2011). LSD test at the 0.05 probability level was used to determine the statistical differences among the different bacteria and fungi isolates.

3. Results

3.1. Bacterial and Fungal Isolates

From different parts of the tomato plants, at total of 28 fungal entities with seven different morphologies were molecularly identified. Also identified were four different pathogenic species of bacteria from 42 isolates (**Table 1**).

3.2. Molecular Identification and Phylogenetic Analysis

The amplification of the 16S and ITS genes were clearly defined in the results. The identification were confirmed

Table 1. Bacterial and fungal isolates with their genebank accession numbers, disease severity scores and ranges.

No.	Pathogens	Genebank Accession number	Isolate name	Diseases caused by	Disease severity score	Disease severity range
1	*Pseudomonas syringae* pv. tomato	KT783474	UPMTPs	Tomato bacterial speck	5	82.3 ± 0.3 e
2	*Ralstonia solanacearum*	KT783476	UPMTRs	Tomato bacterial wilt	5	87.6 ± 0.3 c
3	*Xanthomonas vesicatoria*	KU661975	UPMTXv	Tomato bacterial leaf spot	5	96.6 ± 0.5 a
4	*Xantho monasgardneri*	KP765608	UPMXo1	Tomato bacterial leaf spot	4	71.3 ± 0.6 f
5	*Fusarium oxysporum*	KM039054	UPMT48	Tomato wilt	5	85.6 ± 0.3 d
6	*Fusarium solani*	KM039055	UPMT44	Tomato foot rot	3	55 ± 0.5 i
7	*Fusarium acuminatum*	KM039056	UPMT012	Tomato fruit rot	2	40 ± 0.5 j
8	*Rhizoctonia solani*	KP262071	UUPMT34	Damping off	3	68.3 ± 0.3 g
9	*Phoma destructiva*	KR559677	UPMT08	Phoma blight	5	86.6 ± 0.3 b
10	*Colletotrichum boninense*	KM039057	UPMTS20	Anthracnose	5	89.6 ±0.3 cd
11	*Colletotrichum acutatum*	KT215296	UPMT02	Fruit rot anthracnose	4	64.3 ± 0.3 h

with PCR amplification with primers 16S of all bacteria isolates from the infected tomato plants produced 1400 - 1500 bp amplicon. Consequently, 550 - 650 bp amplicon were produced from PCR amplification with ITS primers of all fungi isolates isolated from the infected tomato plants which confirmed fungi identification.

All bacteria and fungi we obtained clustered together with 95% - 99% relationship with other reference sequences from the gene bank based on the phylogenetic analysis (**Figure 1** and **Figure 2**).

3.3. Pathogenesis Determination

The pathogenicity studies showed that all the fungus (*R. solani*, *F. solani*, *F. oxysporum*, *P. destructiva*, *C. boninense* and *C. acutatum*) and bacteria (*R. solanacearum*, *P. syringae*, *X. vesicatoria* and *X. gardneri*) under investigation had obvious effects on the growth of tomato seedlings. After two weeks, un-inoculated seedlings were free of typical disease symptoms while the seedlings inoculated with the pathogens exhibited them. Fungal isolates were re-isolated from the infected plants but not from the control plants to confirm Koch's postulate. Percent disease severity (PDS) was significantly different among the bacterial and fungal isolates as per the results of indicated by ANOVA (**Table 1** and **Figure 3**).

3.4. Symptom Development on Inoculated Seedlings

5 - 7 days after inoculation, typical disease symptoms were observed on the tomato plants. Small circular spots, which coalesced to form large, irregular lesions on the leaves, were the visible external symptoms of infection by *X. vesicatoria*. These lesions eventually turned dark brown and were water-soaked approximately 3 mm in diameter on the leaves.

The typical symptoms externalized by *X. gardneri* were small circular to angular water-soaked brown lesions. These lesions eventually dried up to form light brown, regular or irregular necrotic lesions and chlorosis soon appeared.

Symptoms caused by *P. syringae* pv. tomato initially started with brown necrotic specks that turned black and encircled with yellow coronas on the leaves and stem.

The symptoms of tomato plants inoculated with *R. solanacearum* started with wilting of the youngest leaves with no foliar yellowing. When the stems of the affected plants were cut and submerged in water, a whitish ooze was evident and the longitudinal sections showed a brown discolouration.

The tomato plants inoculated with fungi exhibited other types of symptoms. *C. boninense* produced small, circular and immersed lesions on the leaves. These lesions eventually turned light brown with orange spore masses in a dark centre. On the other hand, *C. acutatum* triggered dark, sunken and circular lesion on the leaves.

F. acuminatum only showed symptoms of yellowing leaves while symptoms caused by *F. oxysporum* started with yellowing and wilting of the lower leaves followed by the vascular turning to dark brown and being discoloured. *F. solani* inflicted interveinal chlorosis and bleaching of the lamina on the leaves and taproots while reddish brown cortical lesions and vascular discoloration were apparent above and below the lesions.

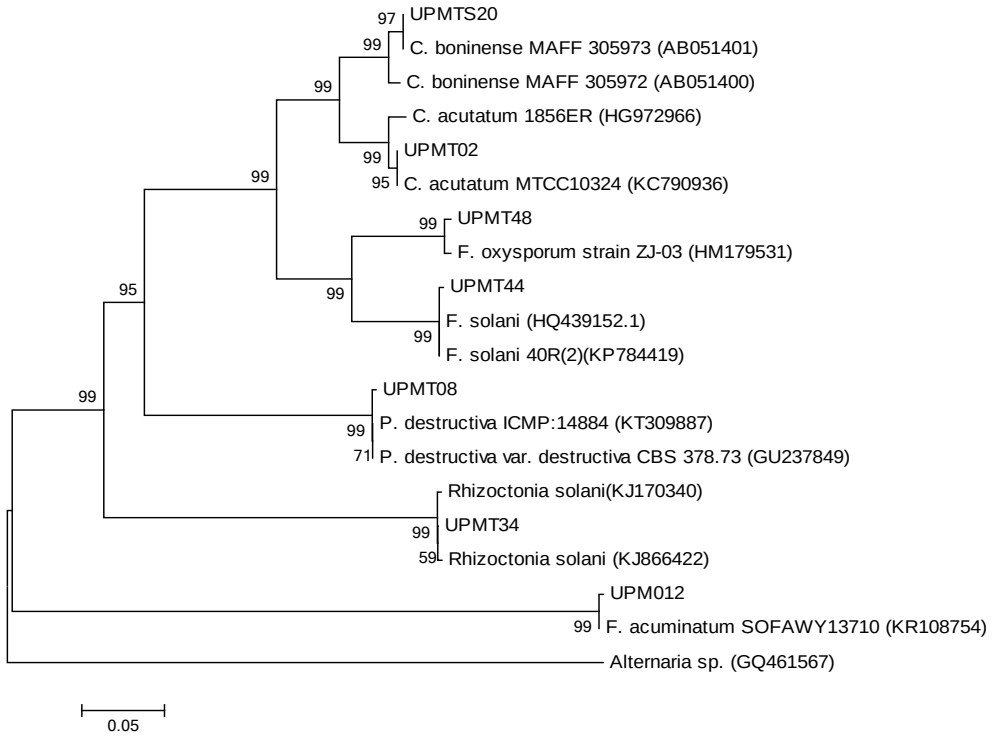

Figure 1. Phylogenetic tree constructed by the neighbor-joining method showing the phylogenetic relationships of all isolated fungi compared with the reference sequences from gene bank.

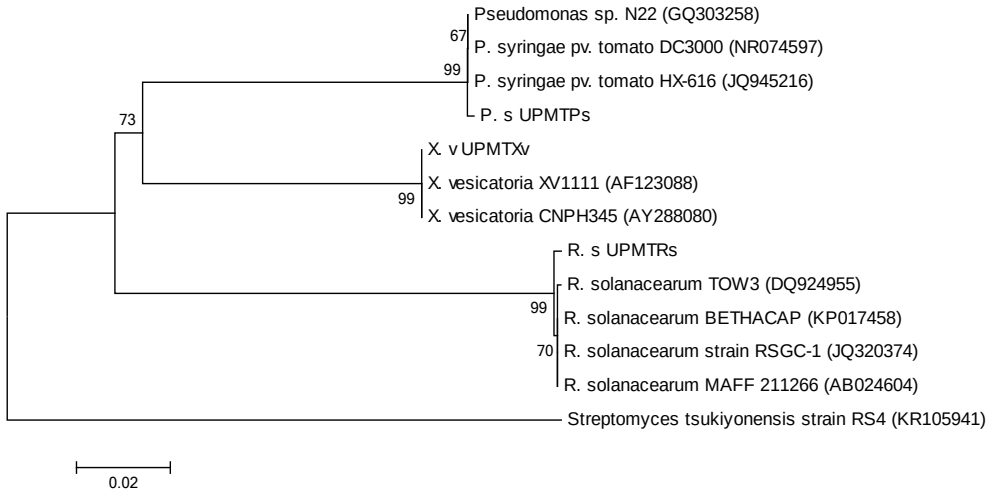

Figure 2. Phylogenetic tree constructed by the neighbour-joining method showing the phylogenetic relationships of all isolated bacteria compared with the reference sequences from gene bank. Based on MEGA 6, neighbour joining was utilised on the phylogenetic tree. On tree nodes, bootstrap values obtained from 500 replications were indicated as percentages. The tree was drawn according to scale with the lengths of the branches in the same units as those of the evolutionary distances used to refer to the phylogenetic tree. The p-distance method was used to compute the evolutionary distances and is in the units of the number of base differences per site.

The symptoms of *P. destructiva* can be identified by small black lesions that appear on leaf surfaces, which are irregular to round in shape, slightly sunken and zonate, stem lesions that elongate and similar to insect punctures or stem scar cracks and all foliar spots develop obvious black pycnidia with hand lens. The symptoms caused by *R. solani* were the hypocotyl, stem cankers and roots turning brown (**Figure 4**).

The pathogenicity tests on tomato seedlings indicated that *X. vesicatoria* and *C. boninense* were highly pathogenic followed by *P. destructiva*, *R. solanacearum* and *F. oxysporum* while *F. acuminatum* and *F. solani* had the least pathogenic.

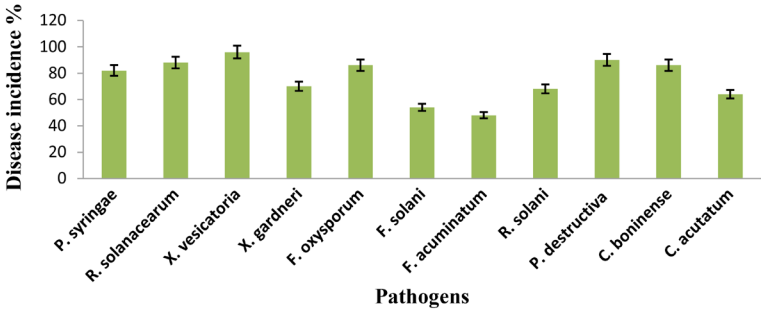

Figure 3. Percentage of disease incidences of bacterial and fungal isolates.

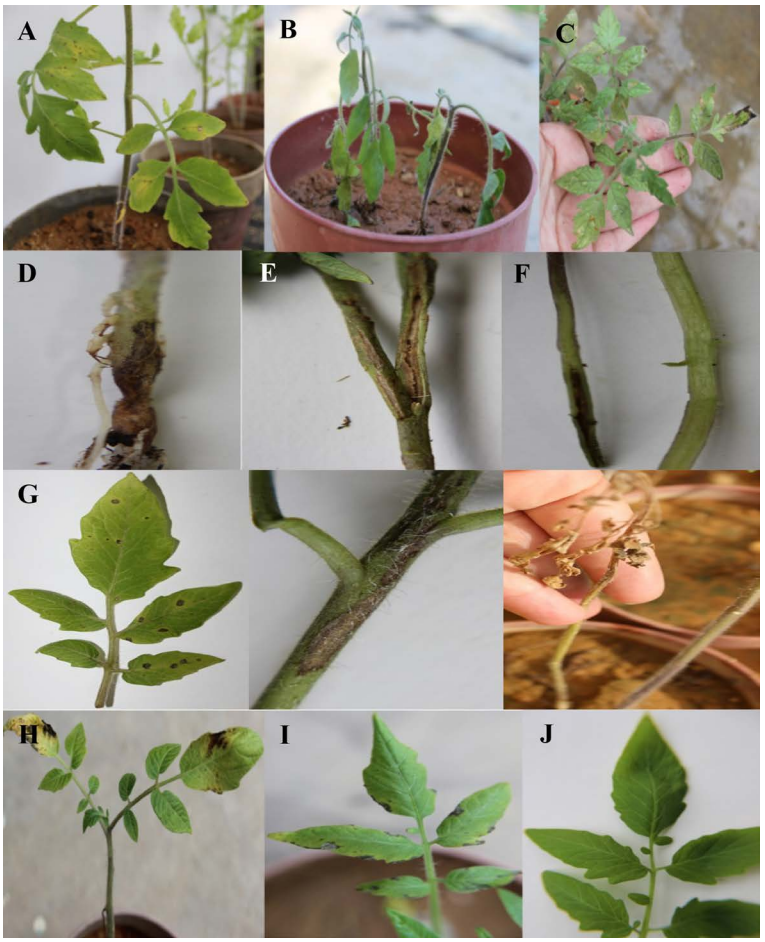

Figure 4. Different symptoms induced by different bacteria and fungi at 2 weeks after inoculation. (A) *P. syringae*, (B) *R. solanacearum*, (C) *X. vesicatoria*, (D) *R. solani*, (E) *F. oxysporum*, (F) *F. solani*, (G) *P. destuctiva*, (H) *C. boninense*, (I) *C. acutatum and* (J) Control.

Re-isolation of the putative fungal and bacterial pathogens was carried out to fulfil Koch's postulates. Colonies that appeared were confirmed to have similar fungal and bacterial morphological characteristics.

4. Discussion

A total of 28 different fungal and 42 bacterial isolates were obtained from 60 samples collected from symptomatic tomato plants at Cameron Highlands, Pahang, Malaysia in this study. Four different bacteria and seven different fungi isolates were identified by molecular techniques to confirm the pathogens. All the selected isolates were pathogenic to tomato plants as confirmed by pathogenicity tests.

The pathogenicity test revealed that the *F. solani* and *F. acuminatum* were the least pathogenic to tomato plants as compared to *F. oxysporum* under test conditions even though the *Fusarium* species were commonly found in tomato plantations. Nevertheless, they still remain a possible risk as some of them still caused symptoms. While causing rot on tomato fruits, *F. acuminatum* was found to be a weak pathogen on tomato seedlings in the present study and these results compare well with the findings of Soekarno and Marhaenis [13].

Chlorosis, leaf wilting and browning of the vascular system were the usual symptoms apparent on inoculated seedlings with *F. oxysporum*. These symptoms were similar to those described byBenhamou *et al*. [14] and Ignjatov *et al*. [15]. Kashif [16] demonstrated that the early symptom was clearing of the ultimate veinlets in the leaflets of infected tomatoes, giving them a "netted" appearance. It could only be seen when viewed with transmitted light. These symptoms were reported byJones *et al*. [17] and Ibrahim [18] to often occur on mature plants after flowering and at the beginning of fruit setting.

Taproots with reddish brown cortical lesions and vascular discoloration above and below the lesions are symptoms of *F. solani* Benhamou and Thériault [19] also mentioned that *F. solani* caused the same symptoms.

Wani [20] has reported that the rots caused by *P. destructiva* are among the most potent diseases faced by tomato plants. Tomato plants were also found to be very vulnerable to *P. destructiva*. This was shown in the pathogenicity tests, which indicated that the most severe disease was caused by *P. destructiva* on tomato seedlings. Similar symptoms with our pathogenicity results were also reported by Wani [20] and Rashid *et al*. [21].

R. solani triggered stem cankers on the inoculated tomato plants. Montealegre *et al*. [22] described similar symptoms caused by *R. solani* where infected mature tomato plants had their roots turn brown and die after a period of time.

We only managed to isolate *C. boninense* and *C. acutatum* even though researchers have reported that *C. coccodes* and *C. gloeosporoides* were the major fungal causes of anthracnose on tomatoes [23]-[25]. *C. boninense*'s symptoms included circular and immersed lesions with orange spore masses in a dark centre, which turn the infected leaves black after three weeks [26].

When plants inoculated with *P. syringae* pv. tomato, the leaves showed black necrotic specks encircled by yellow coronas and the fruits had dark brown to black surface abrasions with a diameter of approximately 2 mm. These symptoms were similar to those mentioned by Louws *et al.* [27], Zhao *et al*. [28] and Ji *et al.* [29]. Other researchers described brown black leaf spots sometimes surrounded by chlorotic margin, dark superficial specks on green fruit and specks on ripe fruit that may become sunken and surrounded by a zone of delayed ripening. If young plants were infected, it would result in stunting and yield loss [30].

Tomato plants exposed to *R. solanacearum* had wilted on foliar with no foliar yellowing. Whitish ooze was evident when the affected plants' cut stems were submerged in water and the longitudinal sections had brown discolouration. These similar symptoms were also mentioned by Huangand Allen [31] and Pradhanang *et al.* [32].

X. vesicatoria caused the leaves to have small circular dark brown spots, which were water-soaked, and approximately 3 mm in diameter. The same symptoms were also mentioned by Jones *et al*. [33], Cavalcanti *et al*. [34] and Kocal *et al*. [35]. Similar symptoms were also observed when tomato seedlings were inoculated with *X. gardneri*, as were reported by Ma *et al*. [36], Quezado-Duval *et al*. [37] and Rashid *et al.* [38]. Previous studies indicated that *R. solanacearum* and *X. vesicatoria* were related to tomato infections in Malaysia [39].

5. Conclusions

Different tomato plant pathogens were collected from various tomato fields in Cameron Highlands, Pahang, Malaysia. Pathogenicity analysis showed that most of the fungi and bacteria isolates were pathogenic towards tomato plants. The pathogen inoculated plants exhibited typical disease symptoms after one week, while un-ino-

culated plants were without such symptoms.

Most of the isolated bacteria and fungi were pathogenic to tomato plants however, *X. vesicatoria*, *R. solanacearum*, *C. boninense*, *P. destructiva* and *F. oxysporum* were the most virulence isolates. *C. boninense*, *P. destructiva* and *P. syringae* pv. tomato were the first reported in Malaysia.

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