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Isolation and Toxicity of *Xanthomonas* spp. in Tomato and Potential of Phage L2.1 against the Pathogen

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The bacterial spot caused by *Xanthomonas* spp. is one of the most common diseases in tomato. In this study, 7 *Xanthomonas* spp. strains were isolated in Lam Dong province, the largest tomato-growing area in Vietnam. All of these strains showed different disease rates in experimental tomato plants. A L2.1 phage specific to *Xanthomonas* spp. was isolated from a tomato field. Morphological analysis indicated that L2.1 had *podovirus* morphology and was classified into the Tectiviridae family. The latent period and burst size of L2.1 were calculated to be approximately 95 min and 4.5 ± 0.27 virions per infected cell. In a test of 13 bacterial strains, L2.1 infected 7/7 *Xanthomonas* spp. strains, while none of the other bacteria tested were susceptible to the phages. *Xanthomonas* spp. was also challenged *in vitro* and was inactivated by L2.1 for 25-27 h in broth at different concentration ratios of phage to bacteria of 0.01, 0.1 and 1.0. Initial characterization of phage L2.1 indicates its potential utility as a biotherapeutic agent against bacterial spot in tomato.

1. Introduction

Tomatoes (*Solanum lycopersicum*) have become an important and common food that provides high nutritional value and has a positive impact on human health (Ha et al., 2021). The tomato growing area in Vietnam is approximately 23,000 ha. One main factor affecting commercial tomato production in Vietnam is the annual cycle of diseases. These diseases not only decrease the yield and quality of tomatoes, but also threaten human health and economic benefits (Singh et al., 2017). One of the most common tomato diseases is bacterial spot caused by *Xanthomonaa* spp. (Potnis et al., 2015). This bacterium can attack on stems, leaves, flowers and fruits. Initially, only small, round to irregular, dark spots appear, possibly surrounded by a yellow halo. The spots are concentrated on the leaf edges and leaf tips. In favorable conditions, the disease can reach 3-5 mm, the leaves are burned, yellow and fall. On the fruit, the disease appears when the fruit is still green, as well as dark spots, turning brown when the fruit is nearly ripe. Although not directly lethal, this damage reduces crop production and quality (Ritchie et al., 2000) and more importantly, it is thought to increase susceptibility to more serious fungal pathogens (Du et al., 2015). The pathogen has been ranked in second place among the top 10 devastating plant pathogenic bacteria (Mansfield et al., 2012). Chemical pesticide usage has been frequently used as a measure of the prevention and treatment of the bacterial spot disease in Vietnam. Improper usage of chemical pesticides can significantly affect the sustainability of tomato farming, agriculture and community health (Louws et al., 2001). Due to these adverse impacts, it is urgently necessary to adopt an alternative solution to chemical pesticides in the prevention and treatment of the disease in tomato (El-Hendawy et al., 2005).

The phage biocontrol is usage of lytic phages to control pathogenic bacteria (Hoang and Pham, 2021). It has been demonstrated that lytic phages are effective and safe when employed to manage bacterial infections in crops, while also being harmless to animals and the environment. Some previous studies have reported that phages can effectively control bacterial spot disease in tomatoes (Obradovic et al., 2004).*Xanthomonas* spp.

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bacteria are very diverse (Morinière et al., 2020), and the capacity of phages to control *Xanthomonas* spp. isolates in Vietnam has not been investigated. In this study, *Xanthomonas* spp. strains were isolated in the largest tomato-growing province in Vietnam. Their toxicity were then examined. A phage specific to *Xanthomonas* spp. was isolated and its characteristics such as morphology, lytic activity, host range were investigated.

2. Materials and Methods

2.1 Isolation of *Xanthomonas* spp.

Twenty-seven samples of tomato plants with the bacterial spot symptoms were collected from tomato growing areas at Quang Loi village, Quang Lap town, Don Duong district, Lam Dong province. Samples were taken from the whole plant, including the stem and leaves, and then placed in a Ziploc plastic bag before being transported to the laboratory. They are stored at 4 °C until used for bacterial isolation. The stem and leaf samples were washed under tap water for 2 min, gently scrubbed the leaf surface by hand to remove large impurities, then blotted the leaf surface with a paper towel. The leaves with typical disease symptoms (irregular round spot, brown or black border on yellowed flesh) were collected using clean scissors. The leaves were immersed in sterile distilled water for about 20 s using sterile forceps to remove any remaining impurities. They were then submerged in 70 % alcohol and rinsed with sterile distilled water. Bacterial spots and surrounding tissue on the leaf surface were separated by clean knife and scissors in the form of small rectangular pieces (0.3 x 0.3 cm) or fine fibers and placed in 1 mL of sterile distilled water. 100 µL of the diluted bacterial suspension was spread on Selective peptone sucrose agar (SPA) medium and incubated at 30 °C for 48 h. SPA medium containing 20 g sucrose, 5 g peptone, 0.5 g K_2HPO_4, 0.25 g $MgSO_4.7H_2O$, and 15 g agar per liter was sterilized at 121°C for 20 min. Single colonies of typical shape and colour of *Xanthomonas* were picked and serially streaked on SPA medium, incubated at 30 °C for 48 - 72 hours. Gram staining was performed according to the method described by Makut et al. (2022). The isolates were confirmed by colony PCR for the *hrp*F gene (Berg et al., 2005). The PCR program was set with a start at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 60°C for 40 s, 72°C for 1 min and a final extension at 72°C for 5 min. Amplified products were separated on a 1 % agarose gel for 30 min at 80 V with a gelled 6x DNA stain. DNA derived from positive isolates confirmed by PCR colonies were sent to the KTEST Company (Ho Chi Minh, Vietnam) for 16S sequencing.

2.2 Toxicity test of Xanthomonas spp. in tomato

All purified isolates were evaluated for their pathogenicity on tomatoes. Six-week-old F1 TN685 tomato plants were used for the toxicity test of *Xanthomonas* strains. After 48 h of incubation, the bacterial strains were collected by centrifugation at 4,000 rpm for 5 min and resuspended in sterile distilled water. The concentration was adjusted to approximately 10^8 CFU/mL before artificial inoculation. Each experimental treatment corresponded to a strain of *Xanthomonas*, and sterile distilled water was used as a control. Ten plants per treatment were inoculated and arranged in a completely randomised design (CRD). Bacterial suspensions were sprayed on all over the plants, petioles and stems by using a mini rebuildable atomizer. Inoculated plants were covered with transparent nylon bags for 24 h to favour bacterial entry through stomata (Osdaghi et al., 2017). After fourteen days, the toxicity of the bacterial strains was evaluated through the disease rate. The disease rate (%) = (Number of diseased plants/Total number of plants in the treatment) × 100. Each treatment was conducted in triplicate.

2.3 Phage isolation

Soil, stem and leaf samples were taken from tomato fields showing the bacterial spot symptoms in Quang Lap commune, Don Duong district, Lam Dong province, Vietnam and transported to the laboratory for bacteriophage isolation. For the soil sample, 10 g of soil was placed in falcon, added 10 mL of distilled water, and 10 % v/w chloroform (Osdaghi et al., 2017). The stem and leaf samples were washed under running water for 2 min, gently scrubbed the leaf surface by hand. The samples were grinded with a mortar. 15 g of the crushed leaf stem sample were collected into a falcon tube containing 15 mL of distilled water and chloroform at the rate of 10 % v/w. The mixture was mixed well by vortexing for 5 min and then centrifuged at 4,000 rpm for 10 min at room temperature to remove the soil layer and the chloroform. The supernatant was collected in 1.5 mL tubes and centrifuged at 11,000 rpm for 5 min to remove bacteria and the remaining chloroform. The obtained supernatant was filtered with a 0.22-µm pore size filter and placed in 9 mL of TSB medium supplemented with 100 µL of *Xanthomonas* culture. The mixture was shaken at 150 rpm for 24 h at 30 °C and then centrifuged at 11,000 rpm at 4 °C for 5 min. The obtained supernatant was passed through a 0.22 µm filter and the filtrate was subjected to plaque assay. A mixture of 100 µL of filtrate and 200 µL of *Xanthomonas* culture was added to 3 mL TSA 0.5 % (maintained at 42 °C) and poured over a 1.5 % Luria–Bertani (LB) agar plate. After overnight incubation at 30 °C, a single transparent plaque was selected from the plate, suspended in SM buffer, incubated

overnight at 4 °C, and passed through a 0.22-µm filter. The resulting filtrate was subjected to the above protocol three times in succession to purify the phage. The morphologies of bacteriophages were examined using a transmission electron microscope at the Vietnam National Institute of Hygiene and Epidemiology.

2.4 Determination of burst size and latent period of phage

The culture of *Xanthomonas* was incubated at 150 rpm and 30 °C until it reached an OD_{600} of 0.1 (approximately 10^8 CFU.mL^{-1}). It was diluted to a density of approximately 10^7 CFU.mL^{-1}. The phage was added at a multiplicity of infection (MOI) of 0.01 (phage:host). This mixture was shaken for 10 min at 150 rpm and 30 °C and then centrifuged at 11,000 rpm for 5 min at 4°C. The pellet was resuspended in the same volume of TSB and incubated in the same condition. A part of the volume was collected and diluted 100-fold in SM buffer on ice every 5 min. The diluted samples were centrifuged at 11,000 rpm for 5 min at 4 °C and the supernatant was used for phage concentration determination using the plaque assay. The burst size and latent period of the phage were determined according to a previously described method (Hoang et al., 2019). The experiment was conducted in triplicate.

2.5 Host range determination of phage

The host range of the phage was evaluated using various bacterial isolates (Table 1), the susceptibility of which was determined using a drop plaque assay. A 200 µl aliquot of an overnight bacterial culture was mixed with 3.0 mL molten 0.5 % (w/v) TSB agar (maintained at 42 °C) and placed onto a 1.5 % (w/v) LB agar plate. After 2 min, each plate received 2 µL of phage stock (approximately 10^9 PFU.ml^{-1}). The plates were incubated at 30 °C overnight and then examined for clear zones on the bacterial lawn.

2.6 *In vitro* control of phage against *Xanthomonas* spp.

The host bacterial culture in TSB was shaken at 150 rpm and 30°C until it attained an OD_{600} of 0.1 (approximately 10^8 CFU.mL^{-1}). It was diluted to a density of approximately 10^7 CFU.mL^{-1}. Next, it was divided into four aliquots, one of which had no phage added (the control) while the others were mixed with the phage at the MOI of 1.0, 0.1, and 0.01. The mixtures were shaken at 150 rpm and 30 °C. The OD_{600} was periodically determined. Each trial was performed in triplicate.

2.7 Statistical analysis

In the toxicity test of *Xanthomonas* in tomato**,** each treatment was conducted in triplicate, and on ten plantlets each time. One-way analysis of variance (ANOVA) was used to process the resulting data using Statistical Package for the Social Sciences (SPSS) version 20 software for Mac. Duncan's multiple range test demonstrated a statistically significant difference between treatments at $p \leq 0.05$.

3. Results and Discussion

3.1 A collection of *Xanthomonas*

A total of 72 isolates expected to be *Xanthomonas* were obtained. The isolates with characteristic yellow, mucoid, and convex of *Xanthomonas* on SPA medium (Figure 1) (Berg et al., 2005).

Figure 1: Colony morphology of Xanthomonas spp. isolated from bacterial leaf spot sample

The results of electrophoresis showed that the amplified product from 7/72 isolates (XC01, XC92, XC151, XC181, XC203, XC233, XC263,) appeared in a band approximately 1.4 kb, corresponding to the expected size of *hrp*F gene (Berg et al., 2005). These results indicated that these isolates were *Xanthomonas* species. These isolates were subjected to a Gram staining test. All isolates belonged to the Gram-negative group, with short

rod-shapes similar to those described for *Xanthomonas*. Results of the 16S rRNA sequencing indicated that all isolates were *Xanthomonas* spp.

3.2 Toxicity of *Xanthomonas* spp. in tomato

In the early stages of the disease, the first visible symptoms of the disease are usually on the leaves of the plant. After 7 d of artificial inoculation, the leaf surface appeared small irregular brown spots (1–3 mm diameter). By day 14, 7/7 strains showed disease on experimental tomato plants. Leaves bearing many spots became yellow, with uneven torn edges (Figure 2b). Leaf spots were coalesced, and occasionally enlarged up to 3 cm in diameter (Figure 2c).

The disease rate of these strains differed significantly. While the XC92 and XC233 strains had an average disease rate of only 76.67 % and 83.33 %, respectively, the remaining 5 strains had a disease rate higher than 85 %. XC151, XC181 and XC203 had the same average disease rate of 86.67 %. XC01 and XC263 had the highest disease rates at 96.67 % and 100 %, (Table 1).

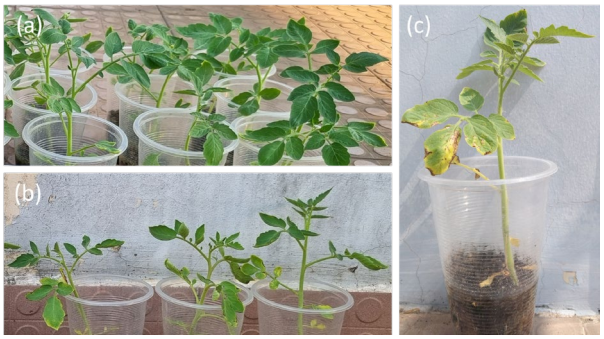

Figure 2: The results of the toxicity test on tomatoes after fourteen days of treatment. a: Control; b: Plants were treated with XC263 strain (disease rate of 100 %); c: After 14 days, the spot disease can reach 3 cm, the leaves are burned, yellow and falling down

Table 1: Toxicity test on tomato plants after fourteen days of artificial inoculation

No.	Strains	Disease Rate (%)
01	*Xanthomonas* spp. XC01	96.67^{ab}
02	*Xanthomonas* spp. XC92	76.67^{c}
03	*Xanthomonas* spp. XC151	86.67^{bc}
04	*Xanthomonas* spp. XC181	86.67^{bc}
05	*Xanthomonas* spp. XC203	86.67^{bc}
06	*Xanthomonas* spp. XC233	83.33^{c}
07	*Xanthomonas* spp. XC263	100.0^{a}

3.3 Lytic activity and morphology of phages

A bacteriophage, named L2.1, was isolated. The phage was presented as a round and clear plaque approximately 1 mm in diameter (Figure 3a). The latent period and burst size of L2.1 were approximately 95 min and 4.5 ± 0.27 phages per infected cell, respectively. As shown in Figure 3b, L2.1 has an icosahedral head approximately 62.80 nm in diameter with no tails. It is classified in the Tectiviridae family (Sanz-Gaitero et al., 2021).

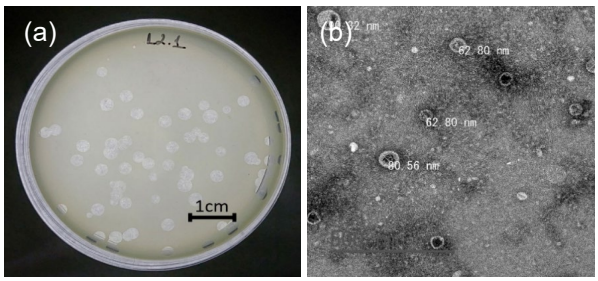

Figure 3: a: Top agar overlay showing plaque morphology of phage L2.1, the scale bar indicates 1 cm; b: Electron micrograph of phage L2.1

3.4 Host range of phage

The lysis capacity of phage L2.1 was described on 13 different strains (Table 2). 7/7 strains of *Xanthomonas* inthe current study showed clear zones. In contrast, three strains of *Xanthomonas oryzae* (LA1+, L5.2, L24) causing bacterial leaf blight in rice did not appear. None of the *Pseudomonas solanacearum* strains (PS003, PS024, PS025) causing bacterial wilt disease on tomatoes tested was susceptible to the phage. Phage L2.1 had a wide range of host bacteria causing bacterial spots in tomatoes.

Table 2. Host range of the phage

No.	Strains	Plaque formation
01	*Xanthomonas* spp. XC01	+
02	*Xanthomonas* spp. XC92	+
03	*Xanthomonas* spp. XC151	+
04	*Xanthomonas* spp. XC181	+
05	*Xanthomonas* spp. XC203	+
06	*Xanthomonas* spp. XC233	+
07	*Xanthomonas* spp. XC263	+
08	*Pseudomonas solanacearum* PS003	-
09	*Pseudomonas solanacearum* PS024	-
10	*Pseudomonas solanacearum* PS025	-
11	*Xanthomonas oryzae* pv. *oryzae* LA1+	-
12	*Xanthomonas oryzae* pv. *oryzae* L5.2	-
13	*Xanthomonas oryzae* pv. *oryzae* L24	-

(+): susceptible; (-): not susceptible

3.5 Inactivation of Xanthomonas by phage

The ability of phage L2.1 to inhibit the growth of *Xanthomonas* in TSB was evaluated. The data in Figure 4 shows the evolution of optical density over time of *Xanthomonas* culture exposed to different initial phage concentrations.

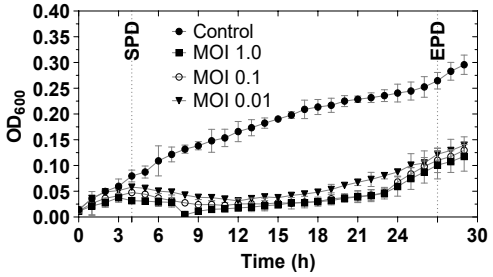

Figure 4: Changes in optical density at 600 nm (OD_{600}) during inhibition of Xanthomonas XC263 by L2.1 phage in TSB at 30 °C. Optical density vs time curves of a control (●) and a phage-inoculated culture (MOI 1.0 (■); 0.1 (O); and 0.01 (▼), respectively) were integrated with the Start Point of Detection (SPD) and End Point of Detection (EPD) of MOI

During the first 4 h, an increase in OD_{600} was observed in both treatments (with and without phage). The results showed that cultures grew to some extent before lysis was apparent. The OD_{600} of the bacterial-phage suspension was always lower, and started to decrease after incubation for 4 h,the OD_{600} of the control continued to increase. This demonstrated that a proportion of host cells were infected and lysed by L2.1. The transparency of the bacteriophage suspension indicated the ability of L2.1 to inactivate host cells, while the turbidity of the control was continuously maintained throughout the experiment. Bacterial inhibition was different at three MOI values of 1.0, 0.1, and 0.01. The phage addition at MOI 1.0 (10^7 pfu/mL) resulted in a very fast decrease in optical density. After only 4 h (the Start Point of Detection (SPD)), the culture was lysed and optical density had closely reached the level of the blank. At MOIs of 0.1 and 0.01 the optical density had a slower effects, started to decrease after 5 h after the beginning of the incubation. The concentration of phage also affected the inhibition time, with high concentration (MOI 1.0), the time of bacterial inhibition lasted 27 h (End Point of Detection (EPD)). At MOI 0.1 and 0.01, after only 25 h, the optical density has increased again (the density of bacteria increases by a log). This led to mention that the time effect of bacterial inhibition depended to a large extent on the

concentration of the phage. The higher the phage concentration, the greater the ability to infect and lysis cells, the longer the inhibition time (Le et al., 2022).

4. Conclusion

The usage of chemical pesticides significantly affects the sustainability of tomato farming and community health in Vietnam. Lytic bacteriophages have attracted serious attention as a tool for the prevention and treatment of bacterial diseases in plants. In this study, seven *Xanthomonas* spp. strains were isolated from Lam Dong province, the largest tomato-growing area in Vietnam. Their toxicity, causing bacterial spot disease, were then examined in tomato plants. A phage specific to *Xanthomonas* spp. was isolated. Its characteristics, including morphology, lytic activity, host range were investigated. It showed potential utility as a biotherapeutic agent against bacterial spot in tomato.

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