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Study of population dynamics of *Clavibacter michiganensis* subsp. *michiganensis* in exposed and buried crop debris

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Abstract

The aims of the current work were to determine the effect of burial depth (0, 6, 12 and 18 cm) on the degradation of diseased crop debris, and survival of the debris-borne bacterial inoculum. This study was carried out in two-locations namely; the Agricultural Research Institute, Tarnab, Peshawar, and the Haripur University, Haripur campus, Pakistan. Results revealed that the debris degraded at faster rate and the number of bacteria per gram of tissue declined more in buried samples than in exposed samples. Moreover, the decline in number of bacteria was more at greater depths than at shallow ones; suggesting that the use of turn-over ploughs after harvest would be helpful in reducing the crop debris-borne primary inoculum, and hence reducing the disease incidence. During a period of 270 days, the mean number of cells of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm)\ gram of exposed stem tissue was reduced from 8.9×10^9 to 6.0×10^5 at ARI, Tarnab, and from 7.93×10^9 to 5.26×10^5 at Haripur. On the other hand, the mean number of cells\ gram of tissue at ARI, Tarnab buried at different soil depths of 6, 12, and 18 cm, decreased from 9.05×10^9 to 3.2×10^3, 8.55×10^9 to 2.9×10^3, and 5.8×10^9 to non-detectable; respectively, after 270 days of burial. Whereas at Haripur; the mean number of cells\ gram of tissue buried at the same depths decreased from 4.28×10^9 to 1.81×10^4, 4.46×10^9 to 2.31×10^3, and 3.82×10^9 to non-detectable, respectively, after the same period.

Keywords: Tomato, *Clavibacter michiganensis*, Soil, Burial depths, Population dynamics, Half-life

1. Introduction

Tomato (*Solanum lycopersicum*) is grown world-wide, and composed of about 7500 varieties (Allen, 2008). Its annual global production was 182.30 million tons in 2017, with yield of 37.6×10^3 kg\ ha. Pakistan produced 0.60 million tons of tomatoes during 2017 (FAOSTAT, 2017), and about 63203 hectares area were grown with different varieties. Average yield of tomato in Pakistan was 9.51×10^3 kg\ ha. Occurrence of different diseases was one of the major reasons responsible for lowering the yield of this crop. Mildews, blights, wilts and cankers were some of the common diseases. Bacterial canker of tomato was one of the most important diseases; first reported from USA in 1910 and probably originated there. Ranges of disease incidence were recorded to be 10-80% with about 20-30% annual losses (Bibi *et al*., 2018).

Clavibacter michiganensis subsp. *michiganensis* (Cmm); is Gram-positive, aerobic, non-motile, non-spore-forming bacterium; which was the causal agent of bacterial canker of tomato, occurring in commercial plantings and home gardens (Seebold, 2008). This disease was seed-borne; where the pathogenic bacterium could survive in the seed for five years, and for more than two years in the infected plant debris (ASTA, 2011). Seebold, (2008) reported that sometimes Cmm could be present in low levels on symptomless plants, multiplying rapidly however, when favorable weather conditions exist. Typical canker symptoms develop at 23-28°C and relative humidity greater than 80% (Xu *et al*., 2012). Through cracks in the stem; the slimy masses of bacteria ooze out to the surface during wet weather, and then spread to leaves and fruits causing secondary infections (Agrios, 2005). According to Kleitman *et al*., (2008); de León *et al*., (2011), primary pathogen inoculum most probably originated each year from residual plant debris in the soil rather than from contaminated seeds.

For the efficient management of the bacterial canker disease, different control measures should be integrated together. Because of the ability of the pathogen to survive in crop debris for a quite long time, we carried out this study to ensure that burial of the diseased crop debris in the soil under our local field conditions; would drastically reduce the number of bacteria per gram of plant tissue over a long period of time.

2. Materials and methods

2.1. Population dynamics of Cmm

An aggressive strain of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm1-MKD1) provided from bacterial culture bank, Department of Plant pathology, The University of Agriculture, Peshawar, Pakistan, was grown overnight at 27°C in nutrient broth yeast extract (NBY) medium, and then incubated with shaking. Bacterial suspension (10^8 cells/ ml) was prepared in 0.85% saline, and then stems of tomato plants were injected with 50µl of this suspension just above the third true-leaf. Plants were kept under green-house conditions (28± 2°C). Two months after inoculation; stem samples (15cm long and 1.5cm diameter each) were selected from inoculated plants and then cut into 1cm pieces. A total of 352 infected stem pieces (176 per location) were used for carrying out the experiment at two different locations namely; one at the Agriculture Research Farm (ARF), Department of Agriculture, University of Haripur, Haripur, and the other at Agriculture Research Institute (ARI), Tarnab, Peshawar. At Haripur, the experiment was started on 10th of May, 2013, and concluded on 7th April, 2014; while at Tarnab, the experiment was started on 13th of May, 2013 and concluded on 10th April, 2014 according to Xu *et al*., (2010).

Initial number of bacterial cells per gram of stems was determined by standard serial dilution method (OEPP/EPPO, 2016). Two 1 cm cross-sections were cut from the ends of each stem. One piece was dried for 7 days at 40°C and used to determine dry weight. The other section was crushed

in 5 ml of sterile phosphate buffer saline (0.05 mM PO_4, pH 7.4); serially diluted to 10^{-6}, 10^{-7}, and then assayed in triplicate BCT medium (Ftayeh *et al*., 2011) plates. Plates were incubated at 28°C for a week. Typical Cmm colonies were counted, and data were converted to number of cells per gram dry weight of tissue.

In order to study the bacterial population dynamics over a period of time, inoculated tomato stem pieces were surface sterilized with 70% ethanol, and then warped in nylon netting. Out of total 176 stem pieces (per each location), 44 pieces were left on soil surface; whereas, the remaining 132 pieces were buried at three different depths i.e. 6cm, 12cm and 18cm (44 pieces were buried at each depth of the soil). These stem pieces were kept in a large sized mud pot. The soil used in this experiment was unsterilized in order to mimic natural field soil (as crop debris remains in field after harvest).

To monitor changes in the population of the pathogen on monthly basis for 11 months (330 days), each stem piece (1 cm) was crushed in a 5 ml of PBS, serially diluted and then plated on Nutrient agar (NA) plates (this has been repeated each month). Plates were incubated at 28°C for 8-9 days. Growing bacterial colonies which showed typical morphology of Cmm such as; colony structure, texture and color) were counted per gram dry weight of the stem tissue. The assay was repeated twice, with four replicate pieces for each soil depth.

2.2. Half-life of Cmm pathogen population

Half-life is defined as the period of time required for a substance undergoing decay to decrease by half. In our study, half-life is the time required for a specific bacterial population to decrease/ reduce to half of its initial population as described by Fatmi and Schaad, (2002). An exponential reduction/ decrease in bacterial population can be described by the following formulas (Gordon, 2018):

$$N_t = N_o\left[1/2\right]^{t/h}$$

Where; No is the initial quantity/ population, Nt is the population that still remains after a time (t), h is the half-life, t is the time. It is to be mentioned that both of "t" and "h" will have the same unit, it might be hours, days, or weeks etc.

2.3. Predicted survival of Cmm population

Predicted survival of the pathogen is the theoretical time required for a specific bacterial population to decrease\ or reduce to zero level; compared to its initial population. This theoretical predicted survival time of Cmm was obtained through extrapolation of the half-life data.

2.4. Statistical analysis

For calculating standard deviation, statistical software STATISTIX® Version 8.1 and Microsoft Excel were used.

3. Results

3.1. Survival of the pathogen in exposed stems

In eleven months (13th May 2013 to 10th April 2014) the mean number of cells of Cmm per gram of stem tissue decreased from 8.9×10^9 to 2.85×10^5 at ARI, Tarnab Peshawar (Table 1), and from 7.93×10^9 to 9.71×10^5 at Department of Agriculture, Haripur University, Haripur (Table 2). The half-life of Cmm in stem tissues placed on the soil surface at Haripur and Peshawar were 25.39 and 22.10 days, respectively (Table 3, Fig. 1). Predicted survival time for the pathogen calculated by the formula of half-life was 914 days for Haripur, and 796 days for Peshawar (Table 3, Fig. 2).

3.2. Survival of the pathogen in buried stems

The mean number of cells of Cmm per gram of stem tissue when buried at a depth of 6 cm decreased from 9.05×10^9 to 3.2×10^3 after 300 days at ARI, Tarnab, Peshawar (Table 1), while from 4.28×10^9 to 2.66×10^3 at Haripur University, Haripur (Table 2).

Average half-lives were 14 and 16.01 days (Table 3, Fig. 1). However, the predicted lives were 504 and 560 days; respectively, for both locations (Table 3, Fig. 2). At the depth of 12 cm, the no. of cells decreased from 8.55×10^9 to 2.9×10^3 after 270 days at ARI, Tarnab Peshawar (Table 1), and from 4.46×10^9 to 2.31×10^3 at Haripur University, Haripur (Table 2). Average half-lives were 12.56 and 12.93 days, while the predicted lives were 452 and 453 days; respectively. At depth of 18 cm, the no. of cells decreased from 5.80×10^9 to 4.9×10^3 after 240 days at ARI, Tarnab, Peshawar; however, decreased from 3.82×10^9 to 7.56×10^2 at Haripur University, Haripur (Tables 1, 2). Average half-lives were 11.9 and 10.78 days (Table 3, Fig. 1), while the predicted lives were 417 and 377 days (Table 3, Fig. 2); for both locations, respectively.

Table 1: Survival of Cmm in tomato stem tissues (cells\ g) under natural conditions, at A.R.I. Tarnab, Peshawar

Days	Soil surface	Buried in soil at different depths		
		6 cm	12 cm	18 cm
0	$8.9\ x10^9\pm14.0$*	$9.05\ x10^9\pm13.5$	$8.55\ x10^9\pm18.5$	$5.80\ x10^9\pm4.0$
30	$6.1\ x10^9\pm15.0$	$2.78\ x10^9\pm1.82$	$7.75\ x10^8\pm1.55$	$4.20\ x10^8\pm1.0$
60	$8.3\ x10^8\pm15.0$	$4.6\ x10^8\pm3.5$	$4.3\ x10^7\pm15.0$	$7.0\ x10^7\pm1.5$
90	$4.85\ x10^8\pm12.55$	$5.8\ x10^7\pm3.5$	$8.05\ x10^6\pm4.95$	$1.8\ x10^6\pm14.0$
120	$4.15\ x10^8\pm12.5$	$5.4\ x10^7\pm2.0$	$2.7\ x10^6\pm1.50$	$6.9\ x10^5\pm13.0$
150	$6.9\ x10^7\pm1.3$	$1.7\ x10^7\pm14.0$	$1.65\ x10^6\pm16.5$	$8.35\ x10^5\pm14.65$
180	$5.85\ x10^6\pm1.55$	$2.9\ x10^6\pm1.5$	$4.35\ x10^5\pm2.05$	$1.84\ x10^5\pm2.82$
210	$3.5\ x10^6\pm12.0$	$8.15\ x10^5\pm4.85$	$4.1\ x10^4\pm1.20$	$1.9\ x10^4\pm18.0$
240	$7.8\ x10^5\pm1.5$	$2.1\ x10^5\pm0.0$	$8.75\ x10^3\pm4.25$	$4.9\ x10^3\pm2.7$
270	$6.0\ x10^5\pm1.5$	$4.8\ x10^4\pm13.0$	$2.9\ x10^3\pm18.0$	0
300	$4.1\ x10^5\pm2.5$	$3.2\ x10^3\pm2.0$	0	0
330	$2.85\ x10^5\pm15.5$	0	0	0

*The standard deviation is raised to the same order of magnitude as the mean.

Table 2: Survival of Cmm in tomato stem tissue (cells\ g) under natural conditions, at Haripur University, Haripur

Days	Soil surface	Buried in soil at different depths		
		6 cm	12 cm	18 cm
0	$7.93 \times 10^{9} \pm 5.03$*	$4.28 \times 10^{9} \pm 3.91$	$4.46 \times 10^{9} \pm 3.72$	$3.82 \times 10^{9} \pm 6.22$
30	$3.64 \times 10^{9} \pm 2.31$	$3.53 \times 10^{9} \pm 3.10$	$2.99 \times 10^{9} \pm 4.09$	$3.53 \times 10^{8} \pm 4.93$
60	$7.53 \times 10^{8} \pm 4.77$	$9.47 \times 10^{8} \pm 1.20$	$2.69 \times 10^{7} \pm 2.11$	$2.60 \times 10^{7} \pm 4.25$
90	$5.99 \times 10^{8} \pm 3.80$	$3.84 \times 10^{8} \pm 4.84$	$1.28 \times 10^{7} \pm 1.76$	$1.50 \times 10^{6} \pm 2.09$
120	$3.56 \times 10^{8} \pm 2.26$	$4.05 \times 10^{7} \pm 4.70$	$9.48 \times 10^{6} \pm 1.35$	$5.32 \times 10^{5} \pm 6.01$
150	$9.71 \times 10^{7} \pm 6.16$	$1.09 \times 10^{7} \pm 1.23$	$3.24 \times 10^{6} \pm 4.49$	$3.25 \times 10^{5} \pm 4.40$
180	$7.04 \times 10^{7} \pm 4.47$	$3.62 \times 10^{6} \pm 4.20$	$8.86 \times 10^{5} \pm 0.11$	$1.96 \times 10^{5} \pm 2.98$
210	$3.48 \times 10^{7} \pm 2.21$	$9.75 \times 10^{5} \pm 1.07$	$3.42 \times 10^{5} \pm 4.94$	$1.12 \times 10^{4} \pm 19.83$
240	$7.53 \times 10^{6} \pm 4.77$	$3.77 \times 10^{5} \pm 4.73$	$1.62 \times 10^{4} \pm 2.33$	$7.56 \times 10^{2} \pm 7.85$
270	$5.26 \times 10^{6} \pm 3.34$	$1.81 \times 10^{4} \pm 2.19$	$2.31 \times 10^{3} \pm 3.39$	0
300	$2.75 \times 10^{6} \pm 1.75$	$6.15 \times 10^{3} \pm 7.86$	0	0
330	$9.71 \times 10^{5} \pm 6.16$	$2.66 \times 10^{3} \pm 3.40$	0	0

*The standard deviation is raised to the same order of magnitude as the mean.

Table 3: Half-lives and predicted survival of Cmm in infected tomato stems, at Peshawar and Haripur, Khyber Pakhtunkhwa, Pakistan

Locations	Half-life (days)				Predicted life (days)			
	Soil surface	Buried at different depths			Soil surface	Buried at different depths		
		6 cm	12 cm	18 cm		6 cm	12 cm	18 cm
Tarnab	22.10	14	12.56	11.9	796	504	452	417
Haripur	25.39	16.01	12.93	10.78	914	560	453	377

* The half-life of Cmm was determined as described by Fatmi and Schaad, (2002). ** The theoretical predicted survival time of Cmm was obtained through extrapolation of the data (half-life and numbers of cells of Cmm).

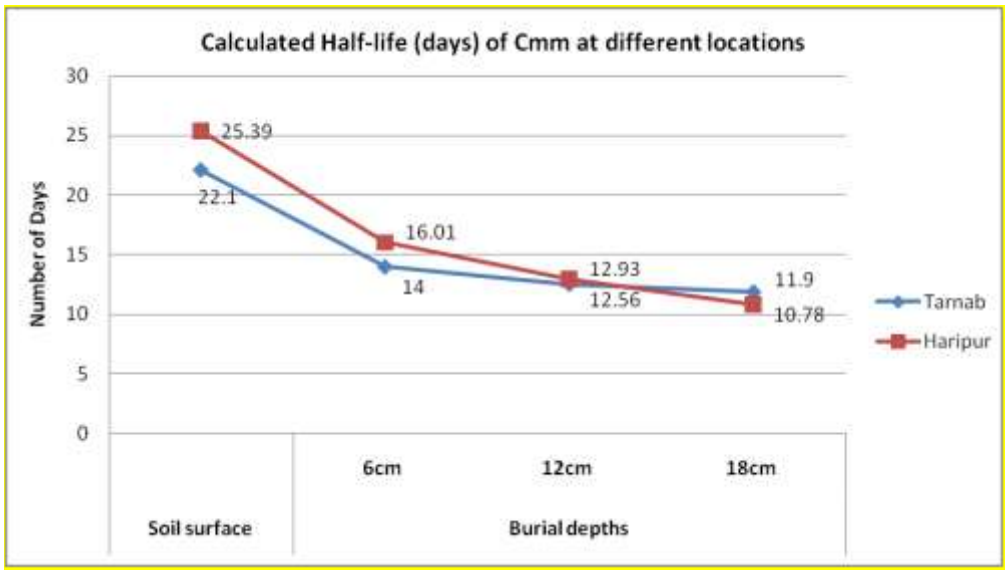

Fig. 1: Half-lives of *Clavibacter michiganensis* subsp. *michiganensis* in infected tomato stems, at ARI Tarnab, Peshawar and Haripur

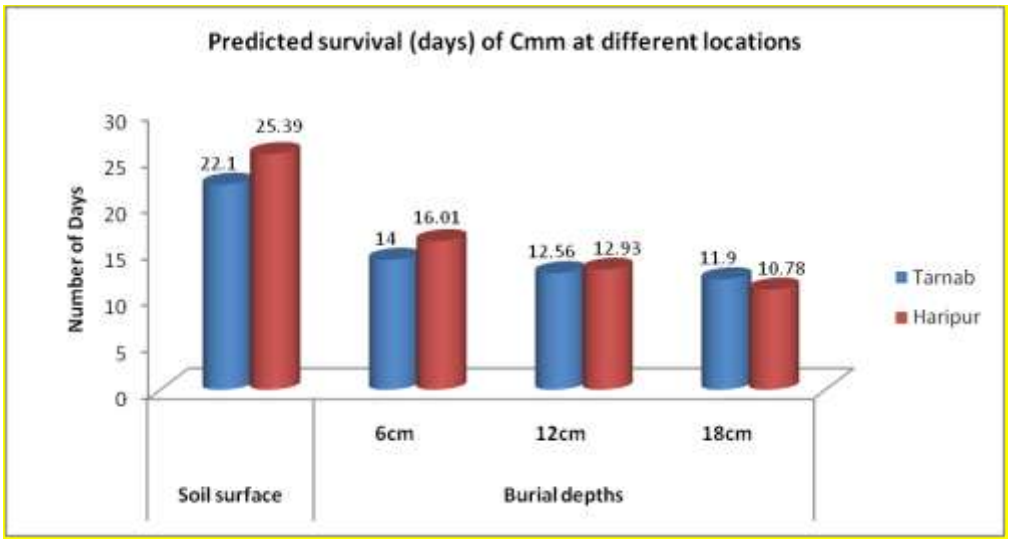

Fig. 2: Predicted survival of *Clavibacter michiganensis* subsp. *michiganensis* in infested tomato stems, at ARI Tarnab, Peshawar and Haripur

4. Discussion

Clavibacter michiganensis subsp. *michiganensis* (Cmm) was found to survive for a quiet long time in infected tomato stems. Survival depended upon the environmental conditions of the location, and on the state whether the stems were left on the soil surface or buried. Sharabani *et al*., (2013); Vega and Romero, (2016); Martinez-Castro, (2018), reported that Cmm can survive in tomato plant debris for several months. Earlier study of Kleitman *et al*., (2008) pointed out that diseased plant debris was considered as the major source of primary inoculum in each growing season.

In the present study, the half-lives of Cmm present in stem tissue placed on the soil surface were 22.10 to 25.39 days at the 2 tested locations; respectively, while the predicted survival time for the pathogen was recorded 796-914 days. Our study also showed that average half-lives were 14 and 16.01 days when inoculated stem tissues were buried at a depth of 6 cm, while the predicted lives were 504-560 days. At depth of 12 cm, the average half-lives were further decreased between 12.56 and 12.93 days, whereas, the predicted lives were 452 and 453 days. At depth of 18 cm, the half-life of the pathogen at the 2 locations further decreased to 10.78 and 11.9 days, and its predicted lives were 377 to 417 days. Our previous findings that Cmm survived for about 30 months in tomato stems placed on the soil surface at Haripur, was similar to the survival times reported by Fatmi and Schaad, (2002). However, our predicted Cmm survival time for buried stems was 12 months; meanwhile it was 18 months in the same study of Fatmi and Schaad, (2002). This variation might be due to climate, soil type and existing competing soil microflora. Cold and heat-shock might also be the cause of this variation; as survival response of this bacterium differs with different surrounding temperature as reported by Ron, (2012); Tambong, (2017).

Our results clearly showed the potential of Cmm to survive for long time in infected tomato stems. Because of this long survival period of Cmm in diseased crop debris, it had been concluded that the use of 'clean' seeds alone will not control the tomato canker disease under field conditions (Fatmi and Schaad, 2002; Yasuhara-Bell, 2013; Mathis, 2015; Yasuhara-Bell, 2015). Previous studies of de León, (2011); OEPP/EPPO, (2016) reported that the bacterium could survives in seeds for up to three years. Cmm might overwinter in the roots and crowns of diseased plants (Talibi, 2011), and this was favored by low soil moisture and temperature (Sharabani, 2014; Sen *et al*., 2015). As revealed by Fatmi and Schaad, (2002); Vega and Romero, (2016), the bacteria could survive on contaminated dry plant tissue for up to 10 years, but not if it was left in contact with the soil. Indeed, the ability of Cmm to compete with soil micro-flora seemed to be low (Amkraz *et al*., 2010; Bouizgarne, 2013).

Conclusions

According to results of current study; we concluded that the Cmm pathogen survived in diseased tomato crop debris for longer periods when this debris was left on the soil surface, than when it was buried. Moreover, diseased tomato debris degraded at a faster rate, and also number of Cmm cells\ gram of stem tissues declined more, when debris was buried at greater soil depths than at shallow ones.

Conflict of interests

The authors declare no conflict of interests.

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