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Effect of Inoculum Density and Methods of Inoculation on the Development of Bacterial Leaf Spot of Bottle Gourd and Pumpkin caused by *Xanthomonas cucurbitae*

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A B S T R A C T

Bacterial leaf spot caused by *Xanthomonas cucurbitae* is one of the important diseases of bottle gourd and pumpkin and is gaining momentum in the sub-tropical zone of Himachal Pradesh. Studies were conducted under artificial inoculation conditions to standardize the inoculum concentration and method of inoculation for the development of disease. The bacterial concentration was standardized with the help of a growth curve of both bottle gourd and pumpkin isolates of *X cucurbitae* with time and a sigmoid curve was obtained in both the isolates in which the bacterial concentration reached its maximum (10^7 cfu/ml) after 96 days of inoculation and then declined. Studies on effect of different inoculum densities of causal bacterium on disease development revealed that an inoculum concentration of 10^8 cfu/ml of both the isolates resulted in minimum incubation period (2 and 4 days) and maximum mean disease severity (38.89 and 42.00%) in both bottle gourd and pumpkin plants, respectively. Among various methods of inoculation with both isolates, syringe method of inoculation resulted in minimum incubation period (2.33 and 3.33 days) and maximum disease severity (46.33 and 37.97 %) in both bottle gourd and pumpkin, respectively followed by pin prick method of inoculation.

Introduction

The family cucurbitaceae is a medium sized family consisting of about 120 genera and more than 800 species collectively known as cucurbits, distributed predominantly in the tropical and subtropical regions of the new and old world (Ajuru and Nmom, 2017). The cucurbitaceous crops are rich in nutritional as well as medicinal value (Khulakpam *et al*., 2015). Gourds, pumpkins and squashes are harvested worldwide in an area of 1.99 million ha with an annual production of 26.48 million metric tonnes (Anonymous, 2016).

In India, bottle gourd and pumpkin are two important cucurbits grown as commercial vegetables crops. Bottle gourd is grown in an area of 1.57 lakh hectare with an annual production of 25.72 lakh tonnes.

The area under pumpkin is 72 thousand hectare with an annual production of 15.82 lakh tonnes (Anonymous, 2017). Like all other crops, cucurbits are also attacked by a wide array of pathogens, out of which bacterial leaf spot caused by *X. cucurbitae* (Bryan) Vauterin *et al.,* (Syn.: *X. campestris* pv. *cucurbitae*) is one of the important emerging pathogen leading to heavy crop losses especially to bottle gourd, pumpkin and squashes (Babadoost and Ravanlou, 2012 and Jarial *et al*., 2015). Significant losses have been reported in different cucurbits due to this disease. In bottle gourd, significant (20-70 %) losses have been reported by Larazev (2009), Jarial *et al.,* (2011) and Basit *et al.,* (2014) while in pumpkin, Salamanca (2014) reported up to 90 per cent losses.

The bacteria presumably persist as epiphytes on the plant surface before they enter the plant via natural openings such as hydathodes, stomata or wounds. Inside the plant tissue, *Xanthomonas* spp. multiply either locally in the intercellular space or colonize the xylem vessels and then spread systemically within the plant (Buttner and Bonas, 2010). An understanding of impact of inoculums concentration on any plant infection is essential to understand any host pathogen interaction. A suitable seedling inoculation method for disease development would be highly desirable for defining conditions that allow optimal infection (Mitchell, 1978).

Many workers have conducted pathogenicity experiments on artificial inoculation of this bacterium by using different methods of inoculations and various inoculum densities (Robbs *et al*., 1972 Taketani *et al*., 1976; El-Hendawy, 1999 Pruvost *et al*., 2008; Babadoost and Zitter, 2009 and Jarial *et al*., 2011), but proper studies on appropriate method of inoculation as well as optimum inoculum density have never been conducted. Therefore, the present investigations were conducted with an objective to standardize the optimum inoculum concentration and method of inoculation for disease development under artificial conditions.

Materials and Methods

Infected leaves of bottle gourd and pumpkin exhibiting characteristics symptoms were brought to laboratory and pathogen was isolated and purified by following standard procedures. The isolated colonies of the associated bacterium were maintained on nutrient agar slants for further experiments and the pathogen was identified on the basis of cultural and biochemical characters as suggested by Society of American Bacteriologists (1957) and Schhad and Stall (1988) and further confirmed by pathogenicity tests.

Standardization of *X. cucurbitae* concentration (cfu/ml) with time

To standardize inoculum density of *X. cucurbitae* (for both isolates) in terms of colony forming units per millilitre (cfu/ml) with time, 72 h old bacterial colonies from NSA slant of both the isolates (bottle gourd and pumpkin) were suspended in 50 ml of NSB to make the starter culture and pour plated to check its concentration (cfu/ml). Simultaneously, 1 ml suspension from this starter culture was inoculated in nine 150 ml capacity Erlenmeyer flasks containing 50 ml nutrient sodium chloride broth and incubated for different durations *viz.,* 0, 6, 12, 24, 48, 72, 96, 120 and 144 hours of incubation. One ml suspension from respective flasks was serially diluted and pours plated on nutrient sodium chloride agar medium to record the colony forming units per millilitre (cfu/ml). A growth curve was further plotted in terms of natural log values of colony forming units per millilitre (ln cfu/ml) with time (h).

Effect of different inoculum density on disease development

An experiment was conducted as completely randomized block design in which bacterial suspension having different concentrations *viz.*, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 cfu/ml of both isolates were adjusted with regard to growth curve obtained in 2.2 and the suspension of each concentration was inoculated on 1 month old bottle gourd and pumpkin seedlings through leaf margin. Inoculated plants were covered with polythene bags internally sprayed with sterile distilled water to maintain the appropriate relative humidity and incubated at room temperature. Each treatment was replicated thrice. The data were recorded in terms of incubation period (days) and disease severity (%). Observations on disease severity were regularly recorded at 2 days interval starting from the day of symptoms development and apparent infection rate (per unit per day) as well as AUDPC were further also calculated as per the formulae given by Van der Plank (1963) and Shanner and Finney (1977), respectively.

Apparent infection rate was calculated by following formula:-

$$\ln x_2/1-x_2 = \ln x_1/1-x_1 + r(t_2-t_1)$$

(Van der Plank, 1963)

where;
r is apparent infection rate (per unit per day) and $x_1$and x_2 are disease proportions at dates t_1 and t_2 , respectively.

AUDPC was calculated as follows:-

$$AUDPC = \sum_{i=1}^{n} \{(Y_{i+1}) + Yi/2\} (t_{i+1} - t_i)$$

(Shanner and Finney, 1977)

where;
Yi and Y_{i+1} are disease proportions at time t_i and t_{i+1}, respectively and n is total number of observations.

Effect of different methods of inoculation on disease development

The effect of different methods of inoculation on disease development was studied by inoculating a standard concentration (10^8 cfu/ml for bottle gourd and pumpkin isolate) of bacterial suspension obtained in 2.2 on 1 month old seedlings of bottle gourd and pumpkin. Following different methods of inoculation were studied to record the data in terms of the incubation period and disease severity and further calculating apparent infection rate and AUDPC.

Inoculation at leaf margins

Drops of standard bacterial suspension were placed on the margins of the leaves of bottle gourd and pumpkin plants with the help of dropper.

Swab inoculation

The leaves of healthy bottle gourd and pumpkin plants were swabbed at both surfaces with sterilized cotton swab soaked in standard bacterial suspension.

Pin prick method

The leaves of healthy bottle gourd and pumpkin plants were pricked with the help of a sterilized paper pin and standard bacterial suspension was swabbed on them with the help of cotton.

Syringe inoculation

Standard bacterial suspension was injected into the mid-rib of the leaves and in the stem

of healthy bottle gourd and pumpkin plants with the help of sterilized hypodermic syringe.

Spray inoculation

The leaves of healthy bottle gourd and pumpkin plants were sprayed with sterilized sprayer containing standard bacterial suspension.

Results and Discussion

The colony characteristics of both pathogen isolates were observed on nutrient sodium chloride agar medium. The colonies were mucoid, circular, smooth textured and yellow in colour (P). On the bases of cultural and biochemical tests, the pathogen was identified as *Xanthomonas cucurbitae.* during pathogenicity tests, incubation periods of 2 and 4 days, respectively were recorded in case of bottle gourd and pumpkin.

Standardization of *X. cucurbitae* concentration (cfu/ml) with time

The concentration of bacteria (both isolates) in culture was recorded to be 10^4 cfu/ml which increased logarithmically with the time and reached its maximum (10^9 cfu/ml) after 72 h of incubation. Thereafter, the concentration of both isolates in the culture started declining and it decreased to 10^7 cfu/ml after 144 h of incubation (Table 1). So, a sigmoid growth curve for bottle gourd isolate and pumpkin isolate was obtained (Fig 1).

Standardization of different inoculum density in relation to disease development

Out of six inoculum densities *(*10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 cfu/ml of both isolates) tested on the plants of bottle gourd and pumpkin. It was observed (Table 2) that when the seedlings of bottle gourd were inoculated with a bacterial suspension having concentration of 1.97×10^8 cfu/ml then shortest incubation period (2.00 days) was recorded which was followed by those inoculated with 0.27×10^9 cfu/ml (2.33 days) and 1.28×10^7 cfu/ml (2.66 days). However, all these three treatments were statistically at par with each other. The longest incubation period (4.00 days) was recorded in seedlings inoculated with a bacterial suspension having inoculum density 3.2×10^4 cfu/ml which was statistically at par with those inoculated with 6.2×10^5 cfu/ml (3.33 days) inoculum density which was further at par with the incubation period of seedlings inoculated with a bacterial suspension having inoculum density of 1.77×10^6 cfu/ml (3.00 days). Significantly maximum mean disease severity (39.65%) was recorded when the bottle gourd seedlings were inoculated with a bacterial suspension of inoculum density 1.97×10^8 cfu/ml followed by those inoculated with a bacterial suspension of concentration 0.27×10^9 cfu/ml (35.70%). However, an inoculum density of 3.2×10^4 cfu/ml incited significantly minimum mean disease severity (24.62%) on the bottle gourd seedlings. Irrespective to the inoculum density, significantly minimum mean disease severity (5.94%) was recorded on 0 day after disease appearance and significantly maximum mean disease severity (54.23%) was recorded after 10 days of disease appearance. It was further noted that there was a significant increase in the disease severity after every two days of observation.

As far as the apparent infection rate was concerned, it was significantly maximum (0.32 per unit per day) in the seedlings inoculated with inoculum density of 1.97×10^8 cfu/ml which was statistically at par with those inoculated with 0.27×10^9 cfu/ml (0.30 per unit per day), 1.28×10^7 cfu/ml (0.30 per unit per day) and 1.77×10^6 cfu/ml (0.29 per unit per day).

However, the apparent infection rate was found to be minimum in the seedlings inoculated with 3.2×10^4 cfu/ml (0.22 per unit per day) which was significantly followed by the apparent infection rate in seedlings inoculated with inoculum density of 6.2×10^5 cfu/ml (0.27 per unit per day).

As far as the AUDPC in the inoculated seedlings was concerned, it was found to be maximum (3.89) in the seedlings inoculated with 1.97×10^8 cfu/ml which did not differ significantly from the AUDPC recorded in the seedlings inoculated with an inoculum density of 0.27×10^9 cfu/ml (3.44) and 1.28×10^7 cfu/ml (3.20).

Interestingly, the AUDPC in the seedlings inoculated with an inoculum density 3.2×10^4, 6.2×10^5 and 1.77×10^6 cfu/ml did not differ with inoculum density of 1.15×10^8 cfu/ml which was statistically at par with those inoculated with 0.19×10^9 cfu/ml (3.82).

Significantly, being minimum (2.46) in the formerly first concentration (3.2×10^4 cfu/ml) followed by second (2.67) and third (2.98) concentration.

Data presented in Table 3 depict the incubation period and disease level in case of pumpkin seedlings. Data reveal that the shortest incubation period (4.00 days) was recorded when the seedlings of pumpkin were inoculated with a bacterial suspension having concentration of 1.15×10^8 cfu/ml which was statistically at par with those in 0.19×10^9 cfu/ml (4.33 days) and 1.02×10^7 cfu/ml (4.33 days).

The seedlings inoculated with bacterial suspension of concentration 2.2×10^4 cfu/ml exhibited longest incubation period (6.00 days) which was statistically at par inoculum density of 1.48×10^6 cfu/ml (5.33 days) and 4.3×10^5 cfu/ml (5.66 days).

From the table, it is evident that irrespective of days of observation, significantly maximum mean disease severity (38.89%) was recorded when the pumpkin seedlings were inoculated with a bacterial suspension having inoculum density 1.15×10^8 cfu/ml which was statistically at par with those inoculated with 0.19×10^9 (38.08%) and 1.02×10^7 cfu/ml (36.43%).

However, an inoculum density of 2.2×10^4 cfu/ml incited significantly minimum mean disease severity (24.41%) on the pumpkin seedlings which was followed by 4.3×10^5 (27.72%) and 1.48×10^6cfu/ml (30.93%). Irrespective to the inoculum density, significantly minimum mean disease severity (6.66%) on pumpkin seedlings was recorded on 0 day after symptom appearance and significantly maximum mean disease severity (57.48%) was recorded after 10 days of disease appearance and there was a significant increase in the disease severity after every two days of observation.

As far as the apparent infection rate was concerned, it was significantly maximum (0.28 per unit per day) in the seedlings inoculated with inoculum density 1.15×10^8 and 0.19×10^9 cfu/ml which was found to be statistically at par with those inoculated with inoculum density of 1.02×10^7 (0.27 per unit per day) and 1.48×10^6 cfu/ml (0.27 per unit per day). However, significantly minimum apparent infection rate (0.22 per unit per day) was recorded in the seedlings inoculated with inoculum density of 2.2×10^4 cfu/ml. As far as the AUDPC was concerned, it was found to be significantly minimum (2.34) in the seedlings inoculated with an inoculum density of 2.2×10^4 cfu/ml followed by those inoculated with 4.3×10^5 cfu/ml (2.68) which was statistically at par with seedlings inoculating of 1.48×10^6 cfu/ml (2.72). However, maximum (3.86) AUDPC were recorded in the seedlings inoculated.

Effect of different methods of inoculation on disease development in bottle gourd and pumpkin

The effect of different methods of inoculation on disease development was studied by inoculating a standard concentration (1.9×10^8 cfu/ml for bottle gourd and 1.15×10^8 cfu/ml for pumpkin isolate) of bacterial suspension obtained in 4.5.2 on 1 month old seedlings of bottle gourd and pumpkin. Data were recorded in terms of the incubation period and disease severity and further calculating apparent infection rate and AUDPC. Data recoded have been presented in Tables 4 and 5.

A perusal of data presented in the Table 4 reveals that among the various methods, the incubation period was minimum (2.33 days) when the seedlings were inoculated by syringe method which was statistically at par with pin prick (2.66 days) and leaf margin (2.66 days) inoculation methods. However, significantly maximum incubation period (7.66 days) was recorded when the seedlings were inoculated with swab inoculation method followed by those inoculated with spray method (6.33 days). As far as, the mean disease severity was concerned, irrespective of the days of observation, it was found to be maximum (46.33%) in syringe method of inoculation which was statistically at par with leaf margin inoculation (43.21%) and pin prick inoculation (42.00%). However, significantly minimum mean disease severity (31.87%) was recorded in swab inoculation method followed by spray inoculation method (36.78%).

Irrespective of methods of inoculation, mean disease severity was minimum (8.15%) on the day of symptom development (0 day) which increased significantly on each subsequent interval of observation and reached maximum (65.41%) after 10 days of disease appearance.

As far as AUDPC in the seedlings of bottle gourd was concerned, it was maximum (4.71) in the syringe inoculation method which was found to be statistically at par with pin prick (4.40) and leaf margin (4.36) methods of inoculation. However, the value of AUDPC in seedlings of bottle gourd was found to be significantly minimum (3.14) which was statistically at par with AUDPC in seedlings inoculated with spray method of inoculation (3.69).

Shortest incubation period was recorded (Table 5) in syringe inoculation (3.33 days) which was statistically at par with pin prick (3.66 days) and leaf margin methods of inoculation (4.66 days). However, maximum incubation period was recorded in swab inoculation (7.33) which was statistically at par with the spray method of inoculation (6.66 days).

As far as disease severity in pumpkin seedlings was concerned, irrespective of days of observation, among the various methods of inoculation, significantly maximum mean disease severity (37.97%) was recorded in the seedlings inoculated with syringe inoculation method followed by pin prick method of inoculation (31.17%) which was statistically at par with mean disease severity in plants inoculated by leaf margin method of inoculation (29.70%).

However, significantly minimum mean disease severity (21.47%) was recorded in the seedling inoculated with swab inoculation. On an average, minimum mean disease severity (9.50%) was recorded on the day of symptoms development (0 day) which increased significantly on each subsequent interval of observation and reached maximum (54.64%) after 10 days of disease appearance with respect to method of inoculation. As far as the apparent infection rate was concerned, it was found to be significantly maximum

(0.30 per unit per day) in seedlings inoculated by pin prick method which was statistically at par with spray inoculation (0.29 per unit per day) and syringe inoculation methods (0.29 per unit per day). However, significantly minimum apparent infection rate (0.17 per unit per day) was recorded in the pumpkin seedlings inoculated at leaf margins whereas, significantly minimum (2.08) AUDPC was observed in swab inoculation followed by spray method of inoculation (2.79).

Based on cultural and biochemical characters, the identity of the pathogen was confirmed to be *Xanthomonas* sp, as the colonies of the pathogen were mucoid, circular, smooth textured and yellow in colour having a diameter of 2-4 mm. The pathogen was found to be Gram–ve, positive for esculin hydrolysis and protein digestion test. These tests were in confirmity with the characters documented by Society of American Bacteriologists (1957) and Schaad and Stall (1988) for genus *Xanthomonas*. The findings were also in accordance with many researchers who worked on *X. cucurbitae* and reported that the bacterium was positive for esculin hydrolysis and protein digestion test and Gram–ve (Lamichhane *et al.*, 2010; Dutta *et al.*, 2013; Ravanlou and Babadoost, 2015 and Sharma, 2016). All inoculum densities tested were able to induce the disease in test plants but, an inoculum density of range 10^7 cfu/ml and above was found to incite significantly higher levels of disease severity and rate of its spread as compared to lower concentrations of bacterial suspension in both bottle gourd and pumpkin isolates.

This indicates that an inoculum density of the range 10^7 cfu/ml is the optimum range of inoculum concentration to incite significant amount of disease in two cucurbits under study. The results were in accordance with findings of many workers who have reported that a bacterial concentration of 10^5 to 10^8 cfu/ml is able to incite pathogenic reaction in different cucurbit hosts. (Pruvost *et al.*, 2009; Lamichhane *et al.*, 2010; Jarial *et al.*, 2011; Babadoost and Ravanlou, 2012; Dutta *et al.*, 2013; Trueman *et al.*, 2014).

Table.1 Population of bottle gourd and pumpkin isolate of *X. cucurbitae* in relation to time

Time (h)	Bottle gourd isolate		Pumpkin isolate	
	cfu/ml	ln cfu/ml	cfu/ml	ln cfu/ml
0	3.2×10^4	10.37	2.2×10^4	9.99
6	6.2×10^5	13.33	4.3×10^5	12.97
12	1.77×10^6	14.38	1.48×10^6	14.21
24	1.28×10^7	16.36	1.02×10^7	16.13
48	1.97×10^8	19.09	1.15×10^8	18.56
72	0.27×10^9	19.41	0.19×10^9	19.06
96	8.4×10^7	18.24	7.3×10^7	18.10
120	3.5×10^7	17.37	2.7×10^7	17.11
144	2.6×10^7	17.07	1.9×10^7	16.75

Table.2 Effect of inoculum density of *X. cucurbitae* on disease development in bottle gourd

Inoculum density	Incubation Period (days)	Disease severity(%) after days of symptoms development							Apparent infection rate (per unit per day)	AUDPC
		0 day	2 day	4 day	6 day	8 day	10 day	Overall Mean		
3.2×10^4	4.00	6.00 (14.04)	14.00 (21.43)	20.00 (26.30)	28.0) (31.62)	36.83 (37.27)	42.89 (40.87)	24.62 (28.59)	0.22	2.46
6.2×10^5	3.33	5.66 (13.62)	13.00 (21.06)	25.33 (30.14)	36.00 (36.83)	43.03 (40.98)	48.32 (44.02)	28.56 (31.11)	0.27	2.67
1.77×10^6	3.00	5.33 (13.16)	14.66 (22.18)	26.00 (30.19)	35.55 (36.28)	45.55 (42.37)	51.94 (46.10)	29.84 (31.71)	0.29	2.98
1.28×10^7	2.66	5.33 (13.16)	22.66 (28.27)	32.22 (34.54)	39.11 (38.62)	48.61 (44.17)	56.38 (48.65)	34.05 (34.57)	0.30	3.20
1.97×10^8	2.00	7.33 (15.67)	22.66 (28.27)	33.58 (35.39)	47.50 (43.54)	58.89 (50.14)	67.95 (55.52)	39.65 (38.09)	0.32	3.89
0.27×10^9	2.33	6.00 (14.04)	24.00 (29.27)	32.78 (34.90)	42.69 (40.77)	50.83 (45.46)	57.89 (49.53)	35.70 (35.66)	0.30	3.44
Over all Mean		5.94 (13.95)	18.50 (25.08)	28.32 (31.91)	38.14 (37.94)	47.29 (43.40)	54.23 (47.45)			
$CD_{0.05}$	0.73	Inoculum density= 2.71 Disease severity = 2.71 Inoculum density × Disease severity = 4.68							0.05	0.72
SE	0.23	Inoculum density= 1.35 Disease severity = 1.35 Inoculum density × Disease severity= 3.32							0.02	0.33

Figures in parentheses are angular/arcsine transformed values

Table.3 Effect of inoculum density on disease development in pumpkin

Inoculum Density	Incubation Period (days)	Disease severity (%) after days of disease development						Over all mean	Apparent infection rate (per unit per day)	AUDPC
		0 day	2 day	4 day	6 day	8 day	10 day			
2.2×10^4	6.00	8.66 (16.74)	12.00 (20.08)	15.55 (23.12)	27.41 (31.55)	36.93 (37.39)	45.94 (42.65)	24.41 (29.49)	0.22	2.34
4.3×10^5	5.66	6.66 (14.79)	13.33 (21.07)	21.11 (27.12)	31.11 (33.84)	40.44 (39.46)	53.65 (47.07)	27.72 (30.56)	0.24	2.68
1.48×10^6	5.33	8.00 (16.07)	16.00 (23.56)	25.55 (30.22)	37.03 (37.43)	44.44 (41.78)	54.65 (47.59)	30.93 (32.78)	0.27	2.72
1.02×10^7	4.33	9.33 (17.75)	25.33 (30.19)	32.88 (34.90)	40.25 (39.29)	49.55 (44.73)	61.22 (51.50)	36.43 (36.39)	0.27	2.96
1.15×10^8	4.00	10.66 (19.03)	18.66 (25.48)	36.91 (37.31)	43.22 (41.06)	56.67 (48.83)	67.22 (55.09)	38.89 (37.80)	0.28	3.86
0.19×10^9	4.33	8.66 (17.09)	24.66 (29.73)	35.95 (36.82)	43.11 (41.09)	53.80 (47.16)	62.31 (52.11)	38.08 (37.32)	0.28	3.82
Over all mean		8.66 (16.91)	18.33 (25.02)	27.99 (31.58)	37.02 (37.37)	46.97 (43.23)	57.48 (49.34)			
$CD_{0.05}$	0.84	Inoculum density = 2.08 Disease severity = 2.08 Inoculum density × Disease severity = 3.58							0.04	0.78
SE	0.27	Inoculum density = 0.73 Disease severity = 0.73 Inoculum density × Disease severity = 1.80							0.02	0.25

Figures in parentheses are angular/arcsine transformed value

Table.4 Effect of different methods of inoculation on disease development in bottle gourd

Inoculation methods	Incubation period (days)	Disease severity (%) after days of disease development						Overall mean	Apparent infection rate (per unit per day)	AUDPC
		0 day	2 day	4 day	6 day	8 day	10 day			
Leaf margins	2.66	6.20 (14.27)	25.00 (29.91)	40.6 (39.60)	52.32 (46.31)	61.11 (51.40)	73.96 (59.31)	43.21 (40.14)	0.34	4.36
Swab inoculation	7.66	9.43 (17.38)	16.00 (22.47)	25.89 (29.66)	36.03 (35.93)	48.93 (44.34)	54.98 (47.86)	31.87 (32.94)	0.30	3.14
Syringe method	2.33	6.30 (14.52)	25.53 (30.03)	45.84 (42.42)	60.55 (51.15)	65.05 (53.79)	74.74 (59.99)	46.33 (41.98)	0.39	4.71
Pin prick	2.66	8.20 (16.57)	27.55 (31.49)	41.22 (39.91)	51.66 (45.94)	57.13 (49.10)	66.22 (54.45)	42.00 (39.58)	0.32	4.40
Spray method	6.33	10.67 (18.93)	19.04 (25.76)	29.99 (33.17)	49.72 (44.82)	54.16 (47.37)	57.14 (49.09)	36.78 (36.52)	0.30	3.69
Over all mean		8.15 (16.34)	22.6 (27.93)	36.72 (36.95)	50.05 (44.83)	57.28 (49.20)	65.41 (54.14)			
$CD_{0.05}$	1.06	Inoculation methods = 3.58 Disease severity = 3.93 Inoculation methods × Disease severity = 6.19							0.12	0.93
SE	0.33	Inoculation methods = 1.26 Disease severity = 1.38 Inoculation methods × Disease severity = 3.09							0.02	0.42

Figures in parentheses are angular/arcsine transformed values

Table.5 Effect of different methods of inoculation on disease development in pumpkin

Methods of inoculation	Incubation period (days)	Disease severity (%) after days of symptom development							Apparent infection rate (per unit per day)	AUDPC
		0 day	2 day	4 day	6 day	8 day	10 day	Overall mean		
Leaf margin inoculation	4.66	13.33 (21.18)	16.16 (23.60)	18.83 (25.55)	28.77 (32.36)	43.33 (41.14)	57.97 (49.58)	29.70 (32.24)	0.17	2.83
Swab inoculation	7.33	11.00 (18.63)	12.20 (19.95)	15.11 (22.29)	20.64 (26.72)	29.99 (32.84)	39.99 (38.97)	21.47 (26.57)	0.22	2.08
Syringe inoculation	3.33	11.06 (18.77)	20.91 (26.96)	34.09 (35.65)	43.80 (41.40)	52.35 (46.33)	65.59 (54.10)	37.97 (37.20)	0.29	3.68
Pin prick inoculation	3.66	6.53 (14.74)	19.33 (25.36)	24.12 (29.36)	35.57 (36.58)	46.00 (42.68)	55.50 (48.14)	31.17 (32.88)	0.30	3.11
Spray inoculation	6.66	5.66 (13.62)	14.66 (22.18)	22.66 (28.18)	30.66 (33.49)	40.22 (39.31)	54.16 (47.38)	28.00 (30.69)	0.29	2.79
Overall mean		9.50 (17.83)	16.65 (23.70)	22.90 (28.20)	31.89 (34.11)	42.38 (40.46)	54.64 (47.63)			
$CD_{0.05}$	1.78	Inoculation methods = 3.10 Disease severity = 3.39 Inoculation methods × Disease severity = 5.58							0.08	0.71
SE	0.55	Inoculation methods = 1.09 Disease severity = 1.19 Inoculation methods × Disease severity = 2.67							0.02	0.32

Figures in parentheses are angular/arcsine transformed value

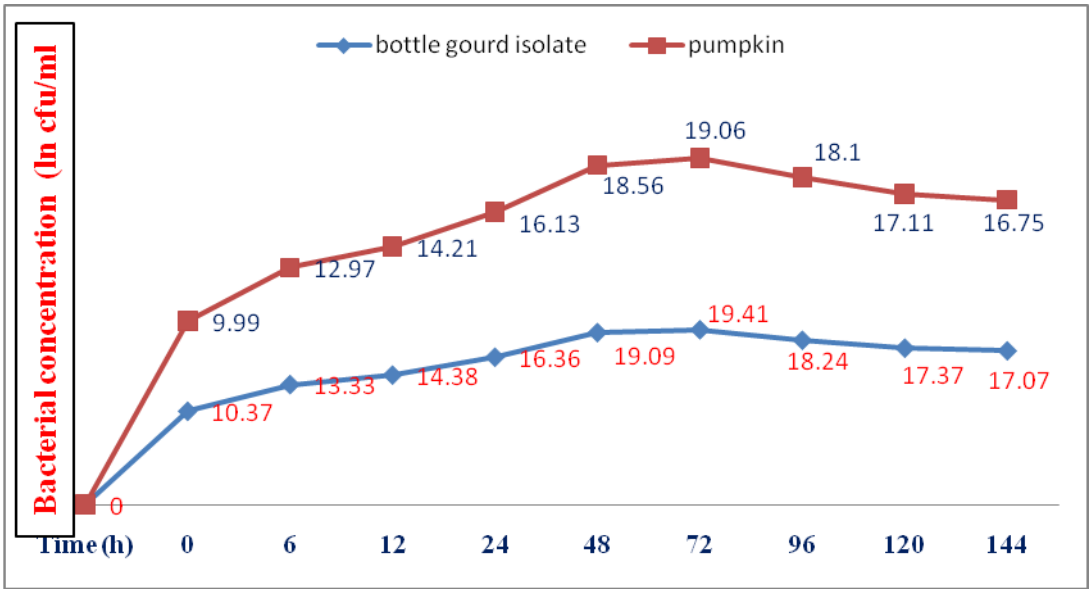

Fig.1 Growth of bottle gourd and pumpkin isolate of *X. cucurbitae* (lncfu/ml) with time

During present studies, syringe inoculation method proved best for the initiation and development of the disease, followed by pin prick and leaf margin methods of inoculation in both bottle gourd and pumpkin isolates of the pathogen. It may be attributed to the fact that the bacterium moves systematically in the plant and thus, the syringe inoculation might have lead to faster spread of the pathogen in the inoculated seedlings resulting in early initiation and faster development of the disease.

In case of pin prick inoculation, wounds were created by pin pricking resulting in easy entry of bacteria and further development of the disease. At leaf margin, hydathodes might have opened the avenues for the entry of bacteria, thus leading to development of disease. In case of swab and spray inoculations the bacteria could enter only through the stomatal openings, thus comparatively lowering the chances of entry of pathogen.

Such type of studies have not been conducted till date on *X. cucurbitae*, so these results cannot be compared with any such work done on *X. cucurbitae*. However, several workers have conducted inoculation method experiments on other *Xanthomonas* spp. The results obtained during present studies are in accordance with Patil *et al.*, (2017) who reported infiltration method to be the most suitable for symptom development by *X. axonopodis* pv *punicae* followed by pin prick method. The results are further supported by the findings of Dhutraj and Soryawansbi (2010) who reported a faster and more pronounced expression of bacterial leaf spot in chilli (*X. axonopodis* pv *vesicatoria*) by injection inoculation and pin prick inoculation as compared to smear and spray inoculation techniques.

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