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Determination of Optimum Incubation Time for the Formation and Quantification of *Xanthomonas axonopodis* pv. *citri* Biofilms on Abiotic Surface

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ABSTRACT: *Xanthomonas axonopodis* pv. *citri* (Xac) is the most devastating pathogen to *Citrus* spp. as well as acid lime and are able to form a biofilm, making it difficult to manage canker disease. Biofilm formation is one of the mechanisms that bacterial communities use to adapt to unfavourable environmental conditions and to survive and colonize in host plants. Hence, the present study aims to investigate the ability of thirty-two isolates of Xac to form biofilms on abiotics surface at different intervals and quantification of biofilm has done. The results revealed that, the biofilms formed strongly adhered to the plates on days one, three, five, seven and nine. Interestingly on day two (48 h) of incubation most of the isolates showed the highest biofilms formation. However, moderate biofilm formation onto the microtiter plates by the isolates were observed on days five and seven, but non-adherence was observed on days one. These strains were grouped into four categories. The isolate which does not formed any biofilm (Xac-23), the isolates with week biofilm formation *viz*., Xac-15 and Xac-26, the isolates with moderately formed biofilm *viz*., Xac-2 and Xac-32, and the remaining isolates *viz*., Xac-1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 31, formed strong biofilm on the walls of microtiter plate compared to negative control. The results concluded that 36-48h is the optimum cultivation period for Xac to form a strong biofilm in microtiter plate and could be used to study biofilm inhibitor studies further.

Keywords: Biofilm, Crystal violet, Ethanol, Microtiter plate, Quantification and *Xanthomonas axonopodis* pv. *citri*.

INTRODUCTION

Numerous biotic and abiotic factors endanger the production of acid lime on a global scale. *Xanthomonas axonopodis* pv. *citri* (Xac), which causes bacterial canker in most of the commercial *Citrus* spp., is one of the main constraints on cultivation in addition to all of these issues. Citrus canker significantly reduces the quality and yields of acid limes by causing defoliation, blemished fruit, and in extreme cases, premature fruit drop. Canker symptoms include surface-penetrating necrotic lesions surrounded by oily, water-soaked margins and yellow chlorotic rings in leaves and fruits (Gopal *et al*., 2001; Schaad *et al*., 2006). For bacteria, a biofilm provides an appropriate haven. Acid lime leaves are directly invaded by Xac through natural openings like stomata or wounds (Das, 2003), and the parasite multiplies in apoplast (Graham *et al*., 2004). After entering the xylem, Xac spreads throughout the tissues, triggering acid-lime defense mechanisms, thickening the secondary wall through lignin deposition, and raising cationic peroxidase levels. According to Gloag *et al*. (2020), biofilms are structural communities made up of collections of bacterial cells encased in a protective polymeric matrix that helps the bacteria survive in harsh conditions and is crucial for infection and host colonisation.

The ability of microorganisms to form biofilms on biotic and abiotic surface causes numerous problems in human health (Cugini *et al.,* 2019) and in other areas of economic importance, including agriculture (Antony *et al*., 2017). In plants, several plant pathogenic bacterial

species have been described to attach to surfaces and form biofilms and such aggregation has been associated with difficulties in disease management (Redondo *et al*., 2015). Biofilms constitute a protected mode for the bacteria that allows them to survive in unfriendly environments, often being difficult to eradicate.

The development of bacterial biofilms is a temporal process that involves moving through various multicellular organisation stages (Monds and O'Toole 2009). Planktonic cells approach a surface and bind to it to begin the process of aggregation and thereby forming biofilms (Stoodley *et al*., 2002). Later, through clonal growth and the recruitment of additional individuals, attached cells form clusters or microcolonies. As the microcolonies grow bigger, macrocolonies start to show up. Bacteria are typically arranged in tower-like groups inside these structures. The exopolysaccharide (EPS) matrix, which is typically made up of water, proteins, exopolysaccharide, lipopolysaccharide, lipids, surfactants, dead cells, and extracellular DNA, holds them together (Strelkova *et al*., 2013). Dispersion, the final stage of this process, occurs when bacteria within the macrocolonies are released (Tolker-Nielsen *et al*., 2000). The multicellular behaviour of biofilms necessitates a specific population size and typically quorum sensing type mechanisms, which entail successful genetic information exchange, intercellular communication, and signal transmission. Specific signal molecules involved in this information exchange ensure that the microbial community responds as a single organism. In order to increase survivability, structured multicellular bacterial communities must be formed and maintained in biofilms, and biofilm formation is linked to virulence in various bacterial pathogens (Janissen *et al*., 2015).

Hibberd (2021) disclosed that *Xanthomonas citri* (Xc) selected from different geographical regions formed maximum mature biofilm on 48 hours of incubation and also observed out of 100 *Xanthomonas citri* isolates, 41 isolates formed high density biofilms. Sabuquillo and Cubero (2021) investigated biofilm formation in *Xanthomonas arboricola* pv. *pruni* (Xap), which causes bacterial spot of stone fruit and almond, on abiotic and biotic surfaces using different microscopy techniques that allowed characterization of the different biofilm stages compared to the planktonic condition and revealed that all Xap strains tested were able to form real biofilms, creating organized structures comprised of exopolysaccharides.Hence, to understand the mechanisms underlying this process in this particular bacterial group, researchers have been studying the biofilm of *Xanthomonas axonopodis* pv. *citri* over the past few years. So, the present study was under taken to determine the optimum incubation time for formation and quantification of *Xanthomonas axonopodis* pv. *citri* biofilms on abiotic surface.

MATERIAL AND METHODS

Bacterial strains and media. The *Xanthomonas auxonopodies* pv. *citri* strains used in this study were isolated from bacterial canker infected acid lime leaf samples collected from three major acid lime growing districts in Andhra Pradesh. These isolates were isolated in the Department of Plant Pathology, College of Horticulture, and all of the isolates were characterized using cultural and biochemical characteristics as mentioned in previous work. All strains were grown for 24 hours at 28° in nutrient broth (NB), a common media for the growth of *Xanthomonas auxonopodies* pv. *citri* isolates, or on plates of nutrient agar medium.

Bacterial growth curve. Overnight bacterial cultures were centrifuged at 4000 rpm for 15 minutes and washed with the same volume of 10 mM $MgCl_2$ before being washed with sterile distilled water. Finally, the bacterial pellet containing cells was suspended in sterile distilled water at a final concentration of OD = 1.0 at 600nm. The bacterial cells were resuspended and sub cultured in nutrient broth media in 1:100 proportions (Bacterial cells: Media). Every 2 hours, a 2 ml volume of each isolate culture was collected to determine the optical density at 600nm (OD_{600}). For each isolate, three replications were kept, and bacterial growth curves were plotted based on the mean OD values.

Biofilm formation and quantification. Each Xac isolate was cultured in flasks on a shaker for 18 hours at 30° (180 rpm) in nutrient agar media, and then diluted to an OD_{600nm} of 0.1 in the same medium. Bacterial adhesion to an inert surface under static conditions, as well as biofilm formation, were measured using a polypropylene 96 well microplate assay with minor modifications to the method described (O'Toole *et al.*, 2000). Each well received 15 µl of an overnight bacterial suspension, which was prepared as described above, and was incubated at 28° under static conditions for the time indicated in each plot. Before and after incubation, spectrophotometric measurements of planktonic growth were taken. Following this incubation period, the media from the static cultures was carefully removed by micropipetting, and the deposited bacteria were incubated for an additional 48 hours in static conditions at 28°.

According to Djordjevic *et al*. (2002), biofilm formation (cell adhesion to the well surface) was quantified spectrophotometrically by staining the adhered bacterial isolates with crystal violet. Wells were carefully rinsed with sterile distilled water and stained for 15 minutes with 0.3 % crystal violet. Excess stain was removed, and the wells were rinsed with sterile distilled water once more. The remaining crystal violet was solubilized in 15 µl of 80 % ethanol and quantified at 570 nm using a microplate reader. The optical density (OD) of each strain was calculated by taking the arithmetic mean of the absorbance of three wells and comparing it to the mean absorbance of negative controls (ODnc). The strains were classified as having no biofilm production (ODs<ODnc), weak biofilm production (ODs≤ODnc), moderate biofilm production (ODnc≤ODs) and strong biofilm production (ODnc<ODs) (Stepanovic *et al*., 2000).

RESULTS AND DISCUSSION

Bacterial growth rate and growth curve. The aim of present study was to determine how bacterial growth was influenced by bacterial aggregation and the biofilm formation in *Xanthomonas axonopodis* pv. *citri*, when nutrient broth used as a basal media. For every 6 h interval up to 60 h of bacterial incubation, the growth of thirty-two *X. axonopodis* pv. *citri* strains was measured for their optical density at absorbance value of 600 nm. Nearly all the 32 strains reached their highest OD_{600nm} between 36 and 42 hours of incubation, remained essentially stable until 48 hours of incubation, and then their concentration of Xac-strains began to decline after 54 hours of incubation (Table 1 and Fig.1).

The aforementioned findings showed that at 48 hours after incubation, all strains had the highest concentration of bacterial cells ranged between 0.745-0.584. The studies by Sabuquillo and Cubero (2021), which provided support for the current study, claimed that the bacterial strains of *Xanthomonas arboricola* pv. *pruni* (Xap) obtained maximum optical density (OD_{600nm}) at 50 hours of incubation and then began to deplete. They also demonstrated that the kind of media used for Xap cultivation affects bacterial growth and bacterial aggregation. These studies of bacterial growth can be used to learn more about the physical requirements for growth as well as the biochemical and molecular pathways of bacteria.

Biofilm formation and quantification. A microtiter plate reader was used to measure the turbidity of the bacterial cells, which were recorded at various time intervals and at an optical density of 570 nm. These plates were further substantiated for the formation of biofilms at 24, 48 and 72 hours after incubation (Plate 1). Following incubation, observations showed that the highest production of biofilms was noted at 48 hours of incubation and varied significantly from 0.59 to 1.44 (Table 2 and Fig. 2). Nearly all the isolates formed biofilm *in vitro* and were divided into four categories based on the OD_{570nm} results by comparing them to the negative control (NC) at OD_{570nm}. The isolate which does not form any biofilm (Xac-23), the isolates with week biofilm formation *viz.*, Xac-15 and Xac-26, the isolates with moderately formed biofilm *viz*., Xac-2 and Xac-32, and the remaining isolates *viz.*, Xac-1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 22, 24, 25, 27, 28, 29, 30, 31 formed strong biofilm on the walls of microtiter plate compared to negative control.

The results of the adhesion assay demonstrated that biofilm formation by the Xac-strains logarithmically increased on day two (48 h) and was significantly higher compared to the next three days. Thus, the findings imply that a significant portion of the bacteria in the plate switched to biofilm on day two. On the second day of incubation, a strong biofilm was formed as a result of an optimal number of bacteria adhering to the surface of the microtiter plate. Hibberd (2021) disclosed that *Xanthomonas citri* (Xc) selected from different geographical regions formed maximum mature biofilm on 48 hours of incubation and also observed out of 100 *Xanthomonas citri* isolates, 41 isolates formed high density biofilms. The present results were also in accordance with Redondo *et al.* (2015); Sabuquillo and Cubero (2021).

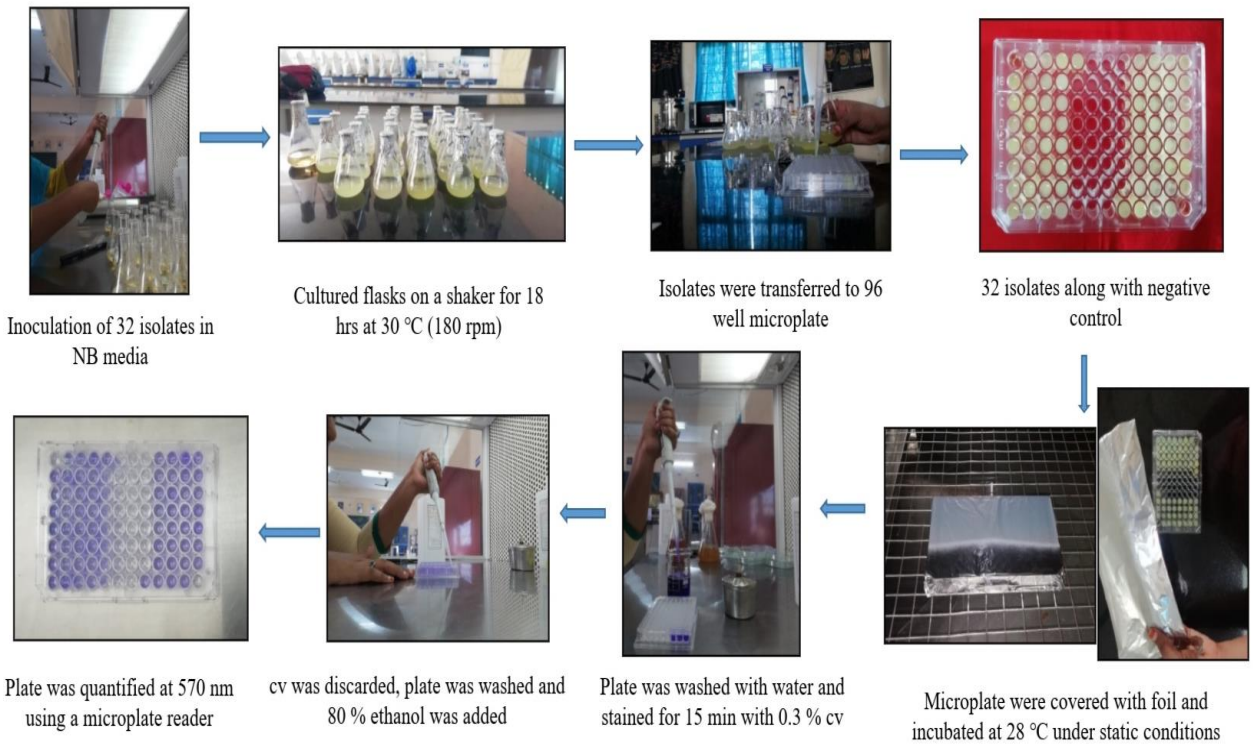

Plate 1. Overview of the procedure of biofilm quantification using 0.3 % crystal violet (cv) stain.

Table 1: Bacterial growth rates ($OD_{600\ nm}$) at every 6 h of time interval up to 60 h of *Xanthomonas axonopodis* pv. *citri* incubation.

Sr. No.	Area of collection	Isolate	Mean of bacterial growth (OD)										
			0hrs	6hrs	12hrs	18hrs	24hrs	30hrs	36hrs	42hrs	48hrs	54hrs	60hrs
1.	Kuppayapalem	XAC-1	0.084	0.106	0.187	0.357	0.528	0.562	0.592	0.589	0.584	0.545	0.392
2.	Dakkili	XAC-2	0.121	0.158	0.243	0.495	0.615	0.653	0.698	0.690	0.676	0.573	0.416
3.	Devulapalle	XAC-3	0.135	0.164	0.188	0.430	0.584	0.584	0.603	0.613	0.596	0.421	0.283
4.	Petluru	XAC-4	0.118	0.136	0.236	0.498	0.623	0.599	0.675	0.662	0.651	0.434	0.321
5.	Peruru	XAC-5	0.146	0.181	0.240	0.517	0.597	0.543	0.572	0.583	0.607	0.396	0.252
6.	Tumaya	XAC-6	0.119	0.146	0.223	0.548	0.685	0.651	0.675	0.684	0.693	0.448	0.295
7.	MPPS Nayanipalle	XAC-7	0.150	0.172	0.268	0.521	0.646	0.683	0.712	0.705	0.696	0.514	0.370
8.	Gudur	XAC-8	0.139	0.156	0.235	0.504	0.630	0.662	0.695	0.682	0.673	0.482	0.347
9.	Kambalapadu	XAC-9	0.108	0.121	0.228	0.490	0.616	0.627	0.638	0.644	0.653	0.505	0.298
10.	Chinnarikatla	XAC-10	0.149	0.166	0.264	0.518	0.640	0.618	0.691	0.685	0.684	0.502	0.352
11.	Pedarikatla	XAC-11	0.157	0.192	0.243	0.536	0.605	0.558	0.592	0.610	0.625	0.359	0.207
12.	Neredupalli	XAC-12	0.124	0.144	0.197	0.491	0.628	0.620	0.655	0.650	0.649	0.459	0.290
13.	Peddhairlapadu	XAC-13	0.162	0.196	0.232	0.541	0.668	0.628	0.653	0.672	0.684	0.382	0.259
14.	Pillolapalli	XAC-14	0.159	0.195	0.248	0.548	0.711	0.731	0.745	0.738	0.729	0.505	0.333
15.	Mallavaram	XAC-15	0.092	0.142	0.225	0.586	0.765	0.832	0.868	0.862	0.847	0.522	0.387
16.	Vellampalli	XAC-16	0.120	0.152	0.236	0.486	0.608	0.644	0.662	0.660	0.659	0.556	0.405
17.	Rachavaripalem	XAC-17	0.132	0.155	0.218	0.524	0.729	0.689	0.732	0.736	0.741	0.514	0.389
18.	Kesavabotlavaripalem	XAC-18	0.135	0.147	0.225	0.521	0.604	0.623	0.632	0.624	0.616	0.529	0.313
19.	Jafalapuram	XAC-19	0.121	0.178	0.283	0.618	0.726	0.597	0.692	0.728	0.744	0.402	0.246
20.	Kothasangatipalli	XAC-20	0.137	0.161	0.202	0.526	0.657	0.623	0.691	0.680	0.678	0.458	0.307
21.	Pendlimarri	XAC-21	0.132	0.172	0.225	0.567	0.715	0.649	0.708	0.712	0.728	0.521	0.315
22.	Kodur	XAC-22	0.108	0.134	0.198	0.459	0.615	0.578	0.597	0.608	0.629	0.352	0.206
23.	Maravaripalli	XAC-23	0.193	0.223	0.293	0.534	0.687	0.702	0.734	0.729	0.722	0.567	0.451
24.	Anantharajupeta	XAC-24	0.145	0.185	0.248	0.569	0.718	0.645	0.701	0.735	0.745	0.524	0.379
25.	Obulavaripalli	XAC-25	0.123	0.156	0.243	0.542	0.686	0.675	0.717	0.709	0.695	0.448	0.251
26.	Yerraguntakota (Y.Kota)	XAC-26	0.120	0.172	0.219	0.523	0.693	0.634	0.681	0.698	0.714	0.487	0.320
27.	Chennarajupodu	XAC-27	0.134	0.158	0.203	0.514	0.647	0.568	0.643	0.662	0.682	0.356	0.232
28.	Kasturivaripalli	XAC-28	0.159	0.209	0.241	0.531	0.688	0.696	0.719	0.720	0.723	0.518	0.304
29.	K. Kandulavaripalli	XAC-29	0.101	0.138	0.170	0.494	0.623	0.638	0.669	0.658	0.640	0.509	0.373
30.	Proddatur	XAC-30	0.131	0.168	0.210	0.497	0.615	0.634	0.663	0.656	0.652	0.521	0.284
31.	Tirupati	XAC-31	0.120	0.153	0.197	0.473	0.672	0.535	0.668	0.673	0.693	0.417	0.227
32.	V.R. Gudem	XAC-32	0.128	0.179	0.304	0.585	0.674	0.628	0.661	0.684	0.695	0.491	0.237
33.	Negative control (NB media)	T_0	0.003	0.003	0.005	0.008	0.008	0.008	0.012	0.012	0.012	0.015	0.015

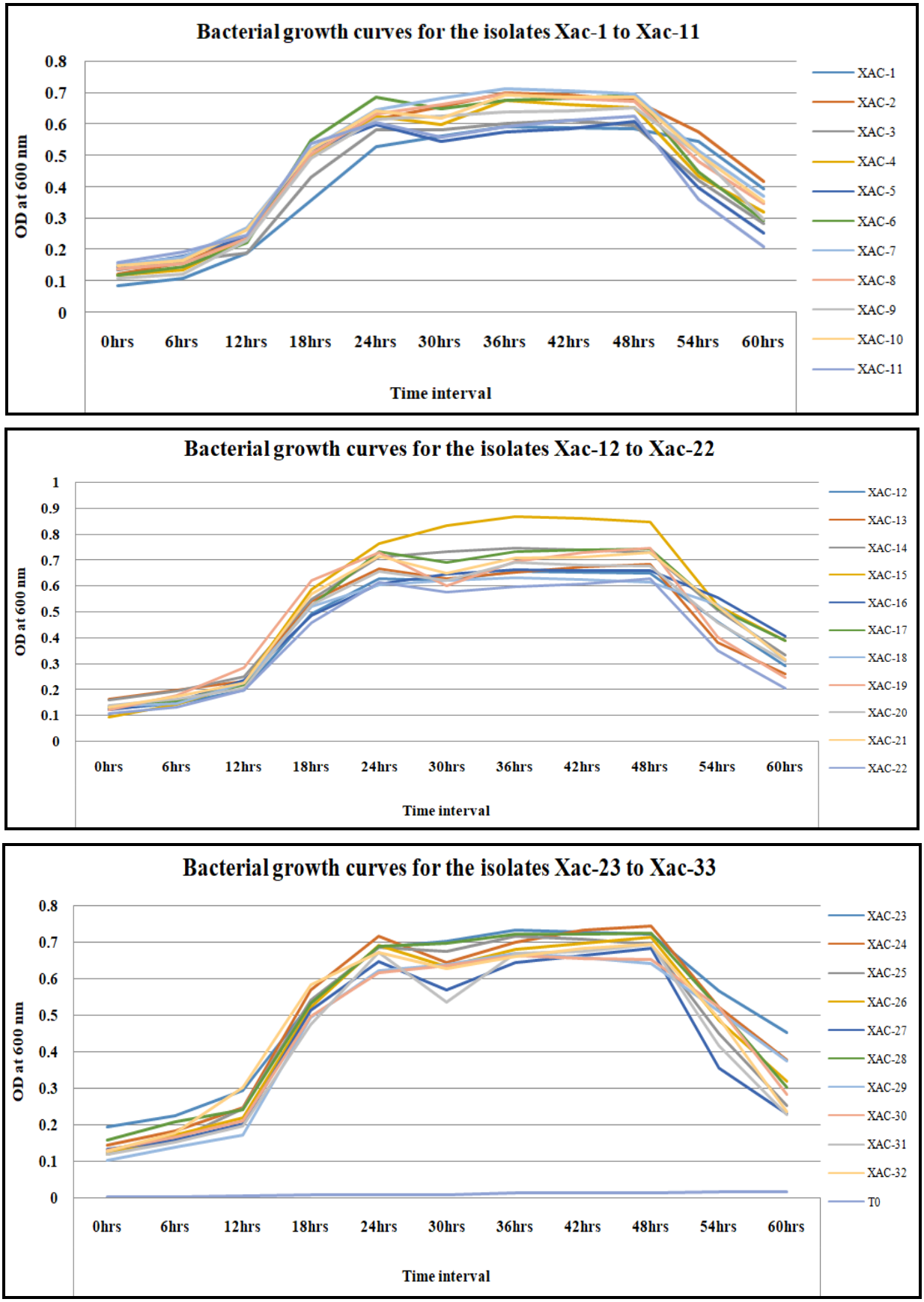

Fig. 1. The growth curves of the isolated *Xanthomonas axonopodis* pv. *citri* strains.

Table 2: Destained biofilm (OD_{570nm}) of inoculated *Xanthomonas axonopodis* pv. *citri* strains at different time interval.

Strain	Destained biofilms of *X. axonopodis* pv. *citri* (OD_{570nm}) values after 24, 48 and 72 hours of incubation			*Category based on biofilm production
	24h	48h	72h	
Xac-1	0.72±0.008	0.96±0.010	0.89±0.003	+++
Xac-2	0.54±0.001	0.65±0.006	0.62±0.001	++
Xac-3	0.98±0.004	1.20±0.14	1.12±0.013	+++
Xac-4	0.64±0.011	0.75±0.002	0.78±0.003	+++
Xac-5	0.83±0.016	0.98±0.004	0.95±0.017	+++
Xac-6	1.16±0.001	1.39±0.012	1.32±0.001	+++
Xac-7	0.62±0.002	0.71±0.002	0.65±0.015	+++
Xac-8	0.86±0.007	0.94±0.006	0.88±0.002	+++
Xac-9	1.09±0.005	1.23±0.012	1.20±0.013	+++
Xac-10	1.26±0.005	1.40±0.022	1.36±0.011	+++
Xac-11	0.80±0.001	0.91±0.008	0.94±0.004	+++
Xac-12	1.01±0.018	1.15±0.002	1.10±0.016	+++
Xac-13	0.66±0.003	0.74±0.002	0.74±0.002	+++
Xac-14	0.81±0.015	0.88±0.002	0.82±0.001	+++
Xac-15	0.56±0.006	0.60±0.010	0.58±0.006	+
Xac-16	1.10±0.004	1.28±0.001	1.17±0.020	+++
Xac-17	0.74±0.001	0.82±0.007	0.80±0.008	+++
Xac-18	0.63±0.001	0.71±0.013	0.62±0.002	+++
Xac-19	0.85±0.002	0.93±0.006	0.95±0.002	+++
Xac-20	0.61±0.003	0.76±0.001	0.69±0.004	+++
Xac-21	1.32±0.025	1.44±0.021	1.41±0.028	+++
Xac-22	0.88±0.004	0.96±0.002	0.90±0.020	+++
Xac-23	0.52±0.002	0.59±0.013	0.59±0.003	-
Xac-24	1.10±0.033	1.17±0.018	1.12±0.018	+++
Xac-25	0.83±0.005	0.98±0.011	0.95±0.017	+++
Xac-26	0.52±0.009	0.62±0.002	0.59±0.002	+
Xac-27	0.80±0.009	0.96±0.006	0.92±0.004	+++
Xac-28	0.64±0.001	0.72±0.005	0.66±0.012	+++
Xac-29	0.78±0.011	0.85±0.003	0.81±0.013	+++
Xac-30	0.85±0.002	0.91±0.004	0.89±0.003	+++
Xac-31	1.16±0.001	1.30±0.005	1.28±0.003	+++
Xac-32	0.57±0.001	0.68±0.009	0.66±0.001	++
NC	0.53±0.002	0.61±0.010	0.62±0.002	-
SE (m)	0.010	0.009	0.011	
SE (d)	0.014	0.013	0.016	

*(-): No biofilm production (ODs<ODnc), (+): weak biofilm production (ODs≤ODnc), (++): moderate biofilm production (ODnc≤ODs) and (+++): strong biofilm production (ODnc<ODs)

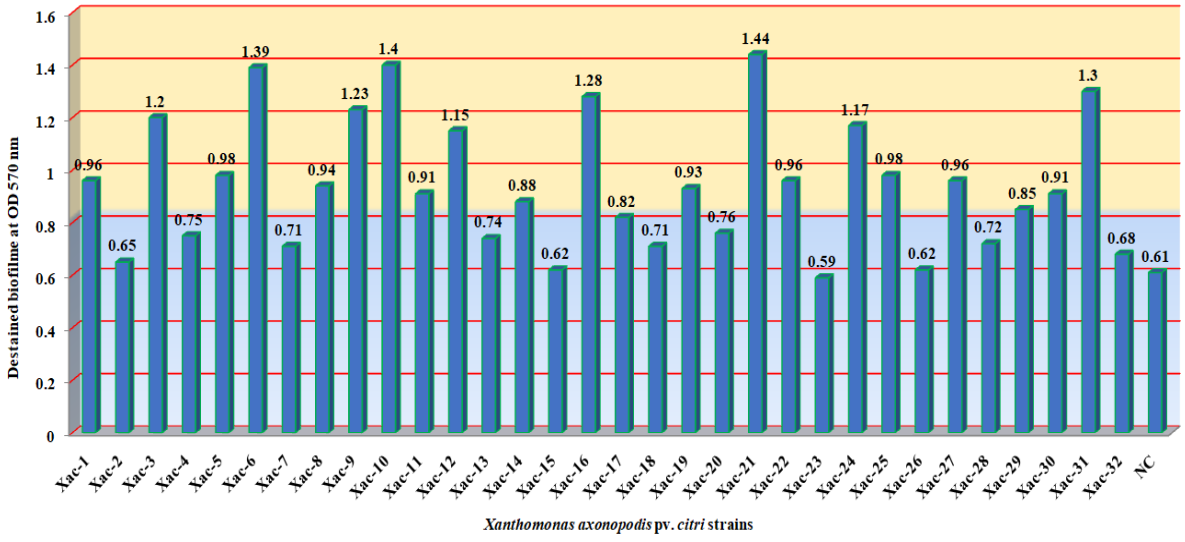

Fig. 2. Destained biofilm ($OD_{570\ nm}$) of *Xanthomonas axonopodis* pv. *citri* strains after 48 h of incubation at 28°.

CONCLUSIONS

Plant pathogenic bacteria can use biofilm formation as a crucial survival tactic in their natural state. To locate and stop the systemic spread of these bacteria through abiotic surfaces, the adherence characteristic of these bacteria must be detected. In this study, we assessed the ability of *Xanthomonas axonopodis* pv. *citri*, a plant-associated bacterium, to form biofilm on an abiotic surface, such as a microtiter plate, and we timed the development of mature biofilms. After 48 hours of incubation, we discovered that Xac strains successfully formed the highest amount of biofilm on a 96-well microtiter plate using nutrient broth, and Xac then started inhibiting the growth of biofilm.

FUTURE SCOPE

This information is required to conduct additional research on biofilms as well as to create novel and targeted control methods for the serious bacterial disease known as acid lime. It is also necessary to conduct additional research to identify the genes responsible for biofilm formation and the role of biofilms in the virulence of *Xanthomonas axonopodis* pv. *citri*.

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Conflict of interest. None.

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