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ORIGINAL PAPER

Virulence Factors Assessment for Some *Xanthomonas campestris* Strains Causing Black Rot in Cabbage

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ABSTRACT

The percentage of the infected area of cabbage leaves was utilized to study the virulence factors of sixteen *Xanthomonas campestris* pv. *campestris* (Xcc) isolates associated with black rot symptoms on cabbage (*Brassica oleracea*). Lipopolysaccharide and adenosine kinase production, cell adhesion, xanthan gum production, and viscosity were all compared for different isolates. The tested strains were divided into three statistical groups, five strains (Xcc4, Xcc7, Xcc11, Xcc13 and Xcc16) gave the highest measurements after incubation, six strains (Xcc1, Xcc2, Xcc8, Xcc10, Xcc14 and Xcc15) had small measurements and five strains (Xcc3, Xcc5, Xcc6, Xcc9 and Xcc12) showed an intermediate value. Finally, increasing the production of lipopolysaccharides, adenosine kinase, cell adhesion, and xanthan gum increases the virulence of *Xanthomonas* strains, whereas the viscosity of the isolates is not considered a factor affecting disease severity or virulence.

Keywords: Cabbage, *Brassica oleracea*, black rot, *Xanthomonas campestris*, virulence, lipopolysaccharides, adenosine kinase, cell adhesion, xanthan gum.

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INTRODUCTION

Xanthomonas (Consisting of *xanthos*, show 'yellow', and *monas*, indicate 'entity') is a huge genus of plant-related Gram-negative bacteria of yellow-tincture and normally rod form with one polar flagellum, it compels aerobes and has an ideal growth temperature of between 25 and 30°C. (Bradbury 1984).

Studies showed, covering a lot of hordes and geographic areas, have recognized the *Xanthomonadales* order as a main order in the microbiome habitats, where it's made up in the middle of 2 and 7% of the bacteria in the microbial group (Bulgarelli *et al*., 2015 and Bhattacharyya *et al*., 2018). But a more precise metagenomic analysis revealed that the abundance of Xanthomonades ranged from under-detection levels to 0.7% in the rhizosphere and soil microbiomes (Souza *et al*., 2016 and Xu *et al*., 2018).

The fundamental method of *Xanthomonas* sp. transition is across contaminated seeds, weeds and stomachic plant ruins (Arias *et al*., 2020). Cells grow epiphytically at first, then enter the horde across any wounds to colonise and settle the parenchyma internally through stomata. (Wang *et al*., 2017). For instance, *X. campestris* pv. *campestris* (Xcc) causes a familiar systemic disease of many plants as black rot, which is characterized by V-shaped chlorotic lesions pervasion from the leaf edges. Elsisi (2017) isolated *Xanthomonas campestris* pv. *campestris* was isolated from leaf margins and blackening of the vascular tissue of showing typical symptoms of bacterial black rot cabbage collected from Qaliobia, Behera and Sharkia governorates in Egypt.

Xanthomonas campestris pv. *campestris* (Xcc), the famed causative disease and Xcc still wily pathogen, actually there are little studies depicting Xcc virulence factors and their function in plant disease. Deciphering the mechanisms can plant-pathogenic bacteria to disband, colonize, and survive on their hosts.

It's known that Xanthomonades extend several virulence factors like the excretion of a broad group of enzymes, various kinds of cell movements, excretion of exopolysaccharide (EPS), bacteria cohesion (Abdalla *et al*., 2021). Several of these malignancy agents affect each other through infirmity advance. For example, disintegration enzymes war plant fortification, reinforce pathogen movement and ease earning of nutrients (Pfeilmeier *et al*., 2016). Moreover, cell movements (flagella- resting on), twitching (flagella- separate) influence cell adherence and biofilm expansion (An *et al*., 2020). Contrast genomic reports revealed that the virulence factors characterized in *Xanthomonas albicans*, exclude for the supersensitive reaction and pathogenicity (Hrp) type three excretion system (T3SS) (Pieretti *et al*., 2012).

Gum genes mass include twelve genes (gumB - gumM) regulate the growth, polymerization, exchange, and exordium of xanthan (Kim *et al*., 2009). Xanthan is the prime EPS produced by ultimate Xanthomonades and it is an important virulence agent of these pathogen (An *et al*., 2020). In addition, xanthan is indispensable for biofilm formatting (Büttner and Bonas, 2010). Biofilms enclose and save Xanthomonades from ecological compression, host protects techniques and antimicrobial compounds, thus, are related to surface permanence and virulence (Büttner and Bonas, 2010).

The goal of this study was to investigate the variance of the pathogenic capability of the sixteen different tested *Xanthomonas* strains and the role of biosynthesis of lipopolysaccharides (LPS), the ability of adhesion on plant surface, the synthesis of adenosine kinase, and the production of xanthan gum and the viscosity.

MATERIAL AND METHODS

Sources of Xanthomonades strains:

The used bacteria in this study were isolated from different aquaponics farms located at Al-Wahat (Giza governorate), Al-Manashy (Giza governorate), Al-Thawra Al-Khadra (Giza governorate), Abo-Sultan (Ismailia governorate), Ismailia (Ismailia governorate) and Al-Sadat (Al-Menoufia governorate). Data in Table (1) show the sources of the 16 *Xanthomonas* isolates, as were isolated from water tank, calendula, cabbage, cherry tomato, basil, purple kale and seed bed from different locations.

Xanthomonas isolates were identified by morphological, biochemical methods and the pathogenicity test (Klement *et al*., 1990) of *Xanthomonas* isolates that were determined on several plants (Eid *et al*., 2019).

Preparation of inoculum of the pathogen:

Bacteria were grown on Yeast extract – Glucose – Chloramphenicol Agar. YGCA media. Cells were rub off from the agar and added 10 mL of sterile tap water to get 10^8 to 10^9 CFU/mL).

Virulence test:

Eight weeks old plants of *Brassica oleracea* were sprayed with standardized inoculum 10^8 to 10^9 CFU/mL) and put in a greenhouse for twelve nights, to permit symptoms to appear. Virulence was estimated by the ***%*** of lesion area on leaves. Five plants for each isolate and five plants for control were tested.

Table (1). Source of *Xanthomonas* isolates and the geographical location.

Xanthomonas isolates	Source of isolates	Geographical location
Xcc1	Water tank	Al Thawra Al Khadra
Xcc2	Water tank	Al-Ismaiellia
Xcc3	Water tank	Al-Mnashy
Xcc4	Water tank	Al-Mnashy
Xcc5	Calendula	Al-Mnashy
Xcc6	Cabbage	Al-Mnashy
Xcc7	Cherry tomato	Al-Mnashy
Xcc8	Cabbage	Ismailia
Xcc9	Cabbage	Abo Sultan
Xcc10	Basil	Abo Sultan
Xcc11	Purple kale	Ismailia
Xcc12	Cabbage	Al-Sadat
Xcc13	Cabbage	Al-Sadat
Xcc14	Cabbage	Al Thawra Al Khadra
Xcc15	Seed bed	Al Thawra Al Khadra
Xcc16	Seed bed	Al Thawra Al Khadra

Chemical characterization of the lipopolysaccharides (LPSs):

Lipopolysaccharides (LPSs) were collected from acetone-dried xanthomonades cells from the aqueous phase (Michael *et al*., 2012). For chemical analyses, the Thio barbituric assay (Brade *et al*., 1983 and Karkhanis, *et al*., 1987) was determined after hydrolyzed LPSs in 1 M HCl. Phosphate content was estimated according to Lowry *et al*. (1954), and hexosamine was estimated according to (Strominger *et al*., 1959). Fatty acids were liberated and derivatized as noticed earlier (Helander *et al*., 1988). Qualitative and quantitative carbohydrate elements were investigated by (Hollingsworth *et al*., 1984). Sugars were analyzed according to (Sawardeker, *et al*., 1965) by gas-liquid chromatography.

Determination of Adenosine kinase (ADK) activity:

Xanthomonas strains inoculated in NYG broth medium for 20h were collected by centrifugation. Adenosine kinase (ADK) activity was determined using a modification of high-pressure liquid chromatography (HPLC) protocol (Zhao *et al*., 1994 and Rajkarnikar *et al*., 2007).

Assay of bacterial adhesion:

Adhesion of sixteen *Xanthomonas* strains was explored by the CV technique (O'Toole and Kolter, 1998).

Xanthan gum production and viscosity from *Xanthomonas* strains:

Xanthomonas strains were grown in YM medium. To xanthan production, 10% (v/v) of cells were mixed to 90 mL of the growing medium was recorded by Ramirez *et al*. (1988).

Statistical analysis*:*

Data were statistically analyzed according to Duncan (1955). LSD test at 5% level of significance was used for comparison between the means of different treatments.

RESULTS AND DISCUSSION

Virulence test:

Sixteen strains of *X. campestris* pv. *campestris* gave yellow, translucent, raised, mucoid bacterial growth on the Nutrient agar (NA) medium A typical Xanthomonades colony from each plate was subculture on YGCA medium and incubated at 28°C for 2 days before inoculation. *Xanthomonas* was scraped from the agar and added in 10 mL of sterile tap water to produce a suspension (10^8 to 10^9 CFU/mL) for the virulence test. Leaves of cabbage were inoculated by giving a tiny cut near the margins. The three new leaves on each plant were inoculated. The disease initiated 6 days after inoculation. All the isolates successfully infected the cabbage seedlings and produced the typical V-shaped lesion with blackened veins but in different proportions (Figure, 1). After confirmation of the pathogenicity of isolates on cabbage, they were designated as Xcc1, Xcc2, Xcc3, Xcc4, Xcc5, Xcc6, Xcc7, Xcc8, Xcc9, Xcc10, Xcc11, Xcc12, Xcc13, Xcc14, Xcc15 and Xcc16.

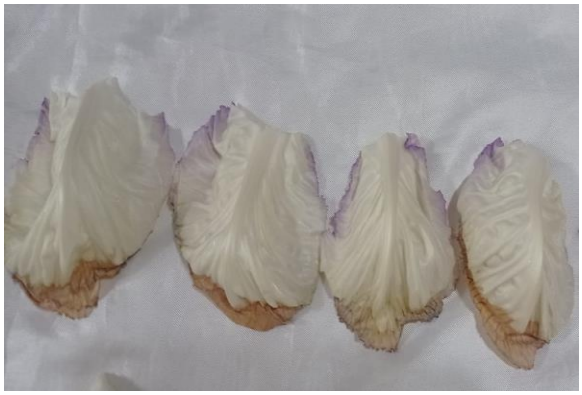

Figure (1): Symptoms of black rot on cabbage leaves

The virulence of isolates was recorded by % of infected lesion area observed in three statistically different groups. Figure (2) shows that, five isolates (Xcc4, Xcc7, Xcc11, Xcc13 and Xcc16) gave the highest area after incubation, six isolates (Xcc1, Xcc2, Xcc8, Xcc10, Xcc14 and Xcc15) had small colony diameter and five isolates (Xcc3, Xcc5, Xcc6, Xcc9 and Xcc12) showed an intermediate value.

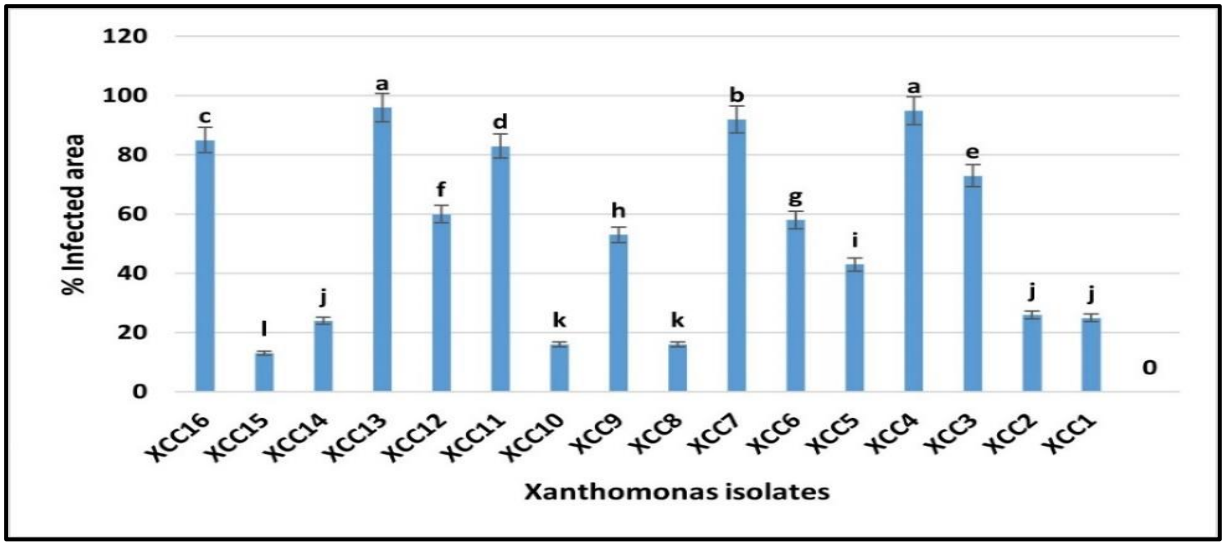

Figure (2): Percentage of infected area caused by sixteen *Xanthomonas campestris* strains as a sign of virulence of each strain (Means with the same letter are not significantly different).

Lipopolysaccharides analysis:

Chemical characterization of the LPSs.:

LPSs refined from sixteen isolates were described chemically, conclude carbohydrates and phosphate in the ratio recorded in Table (2). Glucose, rhamnose, and mannose were current in various quantity in every Lipopolysaccharide. The test of sugars detected that galactose was truant in total Lipopolysaccharides. As estimated by GC mass spectrometry, this material created amongst, a notable mass fragment at mlz 219, signalize that, galactitol peak was limited derives from galacturonic acid. The fragment at mlz 219 and other fragments that specified carboxyl lessening at C-6 were truant from the mass spectra of glucitol and mannitol hexa acetates. Thus, the uronic acid motif of *Xanthomonas* sp. LPS was special galacturonic acid. In each of the xylose- consist of LPSs, a 6-deoxy-3-O-methyl hexose was characterized, a 3-amino-3,6-dideoxyhexose was determined. Significantly, heptoses were not found in LPSs.

The chemical analysis of the lipopolysaccharides from sixteen strains of *Xanthomonas* sp. revealed that they were clustered into 3 main groups: those with a xylose- and 3-0-methyl-6-deoxyhexose-containing, 3-amino-3,6- dideoxyhexose and fucose (Table 2). Based on LPS sugar structure, Xanthomonades have previously been severely distinguishing oneself into 3 prime groups consist of either xylose or fucose or none these sugars (An *et al*., 2020). Our LPS analysis indicated that each xylose-containing LPS had a trace of 3-0-methyl-6-deoxyhexose, which had not previously been reported for Xanthomonades. Nevertheless, since 0-methyl sugars are found in the LPSs of most bacterial populations, the total value of these sugars is unknown. A 3-amino-3,6-dideoxyhexose found in the LPSs of *Xanthomonas campestris* pv. *vesicatoria* and XCC has previously been identified as a major component of the phenol soluble LPS of *Xanthomonas campestris* (Steffens *et al*., 2016).

Table (2): Compositions of LPSs from different *Xanthomonas* strains.

	Lipopolysaccharides (μ mol/mg)										
Xanthomonas strains	Hexose amin	TBA	Phosphate	Galacturonic acid	Rhamnose	Mannose	Glucose	Fucose	Xylose	3-o methyle-6 deoxyhexose	3-Amino-3,6-dideoxyhexose
Xcc1	0.32	0.05	0.10	0.15	0.11	0.09	0.09	0.2	0.5	0.0	0.0
Xcc2	0.38	0.04	0.20	0.13	0.18	0.03	0.05	0.1	0.5	0.0	0.0
Xcc3	0.42	0.09	0.98	028	0.27	0.17	0.18	0.0	0.0	0.0	0.02
Xcc4	0.58	0.12	1.43	0.45	0.35	0.25	0.28	0.3	0.2	0.1	0.02
Xcc5	0.46	0.08	1.01	0.29	0.23	0.19	0.14	0.2	0.0	0.1	0.1
Xcc6	0.48	0.09	0.93	0.27	0.21	0.13	0.17	0.0	0.0	0.0	0.0
Xcc7	0.53	0.17	1.94	0.37	0.37	0.29	0.22	0.1	0.1	0.2	0.05
Xcc8	0.32	0.06	0.36	0.14	0.12	0.06	0.07	0.0	0.0	0.0	0.0
Xcc9	0.45	0.07	1.01	0.26	0.20	0.15	0.19	0.1	0.0	0.1	0.1
Xcc10	0.33	0.5	0.12	0.16	0.17	0.02	0.08	0.3	0.0	0.0	0.1
Xcc11	0.51	0.19	1.33	0.35	0.42	0.13	0.24	0.3	0.2	0.1	0.0
Xcc12	0.41	0.08	1.1	0.21	0.22	0.15	0.17	0.0	0.0	0.0	0.0
Xcc13	0.57	0.18	1.58	0.33	0.39	0.18	0.23	0.3	0.0	0.1	0.04
Xcc14	0.30	0.3	0.52	0.10	0.19	0.05	0.06	0.0	0.0	0.0	0.0
Xcc15	0.36	0.5	0.82	0.12	0.12	0.03	0.09	0.0	0.0	0.0	0.0
Xcc16	0.56	0.17	1.37	0.39	0.45	0.17	0.29	0.2	0.2	0.2	0.05

Adenosine kinase (ADK) activity and adenosine content in *Xanthomonas campestris* isolates:

The intracellular ADK activities of sixteen strains of *Xanthomonas* sp. were determined and contrasted. ADK actions were determined by the HPLC assay. As shown in Fig. (3), strains (Xcc4, Xcc7, Xcc11, Xcc13 and Xcc16) had a high value of ADK activity that was (73.2, 78.4, 72.3, 77.2 and 75.5) (p mol AMP min^{-1} mg $protein^{-1}$), respectively, and strains (Xcc1, Xcc2, Xcc8, Xcc10, Xcc14 and Xcc15) had a low level of ADK level was (42.3, 48.5, 40.7, 38.8, 37.5 and 30.7) (p mol AMP min^{-1} mg $protein^{-1}$) respectively. Adenosine was noticed by HPLC Tanique. Data offered, show that adenosine quantity in the strains (Xcc4, Xcc7, Xcc11, Xcc13 and Xcc16) was highly enhanced (Fig., 4).

Adenosine kinase is a purine-saving enzyme that stimulates the adenosine phosphorylation (Zhulai *et al*., 2022). ADKs belong to the phosphofructokinase (PfkB) group of carbohydrate and nucleoside kinases, a group of concerning enzymes that contains fructokinase, ribokinase, and hexokinase (Park and Gupta, 2008). ADKs from bacteria had been recognized from gram-positive bacteria (Long, *et al*., 2003 and Rajkarnikar *et al*., 2007). ADK described from a gram-negative microbe, the adkXcc identified from Xanthomonades isolates was recorded newly in this study. Two *X. campestris* pv. *campestris* isolates, ATCC 33913 and B100, have been estimated inclose a supposed protein homologous to ADK of Xcc. Amino acid sequence in pairs. Alignments displayed that the prophesy proteins for two strains of *Xanthomonas* sp. which are shown as a sugar kinase and a supposed carbohydrate kinase (da Silva *et al*., 2002 and Vorholter *et al*., 2008). It is indicated about it, prior array-based relative genome hybridization test elucidated that ADK Xcc is extremely preserved in total of the 18 other Xanthomonades strains examined (He *et al*., 2007). Data indicated that the ADK enzyme is excessively disseminate amidst Xanthomonades bacteria.

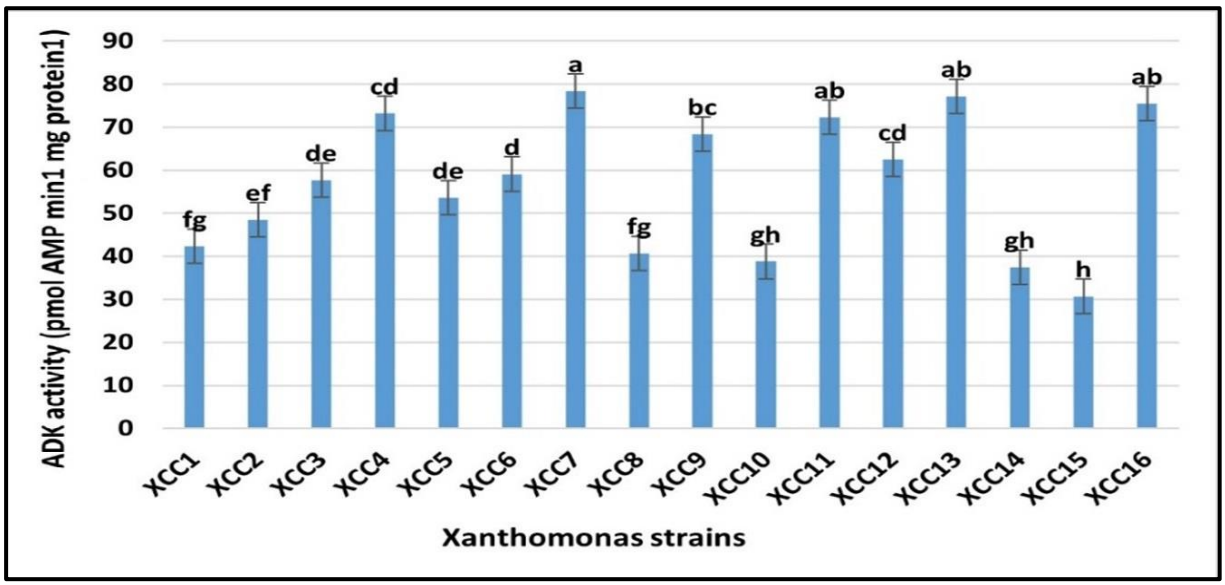

Figure (3): Adenosine kinase (ADK) activity in Xanthomonades strains Adhesion of *Xanthomonas* strains (Means with the same letter are not significantly different).

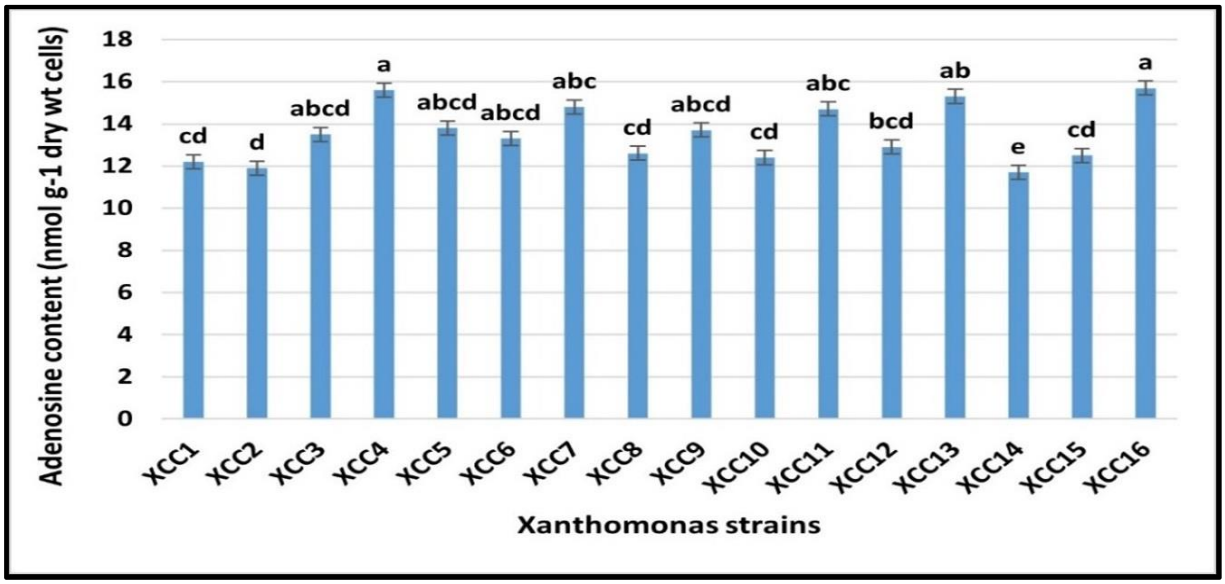

Figure (4): Adenosine content in Xanthomonades strains Adhesion of *Xanthomonas* strains (Means with the same letter are not significantly different).

Cell adhesion:

Cell adhesion is one of the important virulence factors for the emergence of the disease on the surface of the plant and the internal parts of the plant (An *et al*., 2020). Bacterial adhesion to abiotic surfaces was investigated using the CV technique (O'Toole & Kolter, 1998) in a 96-well microtiter polystyrene plate, 200 µl of each bacterial suspension (OD600 0.1) were used per well and microplates were incubated at 28 °C for 48 hr. with no agitation. After this time, medium was gently removed, and wells were washed twice with 0.9% (wt/vol) NaCl and air-dried. Then, each well was stained using 0.1% (wt/vol) CV solution in water for 30 min at retention (RT). Finally, the unbound CV was washed twice with 0.9% (wt/vol) NaCl and wells were air-dried. CV was dissolved by adding 200 µl of ethanol: acetone solution (80:20 vol/vol) to each well. After 10 min of incubation at RT with gentle agitation, absorbance was measured at 570 nm (CV570). The adhesion value was normalized to the number of non-adhered cells at OD600 (CV570/OD600). Data showed that *Xanthomonas* strains (Xcc4, Xcc7, Xcc11, Xcc13 and Xcc16) presented cell adhesion values much higher than *Xanthomonas* strains (Xcc1, Xcc2, Xcc8, Xcc10, Xcc14 and Xcc15) (Figure 5). The obtained results are in harmony with Mielnichuk *et al*. (2021) who recorded the ability assessment of 5 strains of *Xanthomonas* to fix to surfaces and reported that *Xanthomonas sacchari* (XS) offered remarkably increase of cell adhesion than *Xanthomonas albilineans* (Xa).

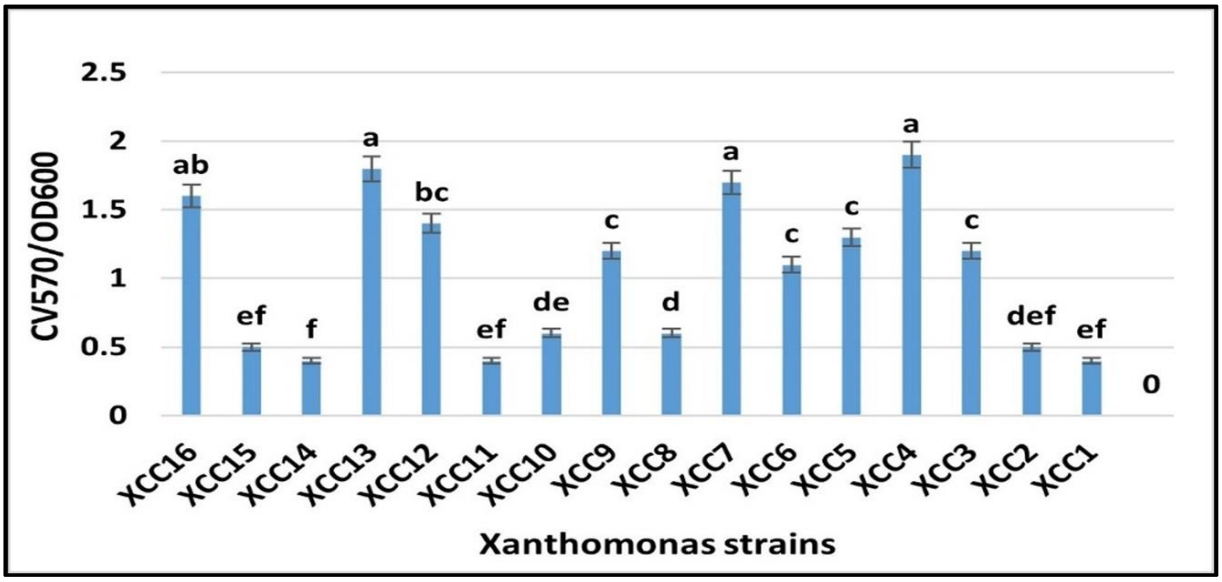

Figure (5): *Xanthomonas* adhesion on leaves surfaces. Different letters point significant differences based on Tukey's test (one-way analysis of variance, *P*< *.0001*) (Means with the same letter are not significantly different).

Xanthan gum production and viscosity from *Xanthomonas* strains:

Xanthan rate was between 6.5 to 14.6 g Kg-1 and viscosity varied from 175 to 575 cP. (Figures 6 and 7). Although low virulence was detected from strains (Xcc1, Xcc2, Xcc8, Xcc10, Xcc14 and Xcc15), the strains produced a solution viscosity ranged from 395 to 524 cP. Data were presented to linear regression analysis and less correlation noticed (r2<0.4) between virulence and viscosity.

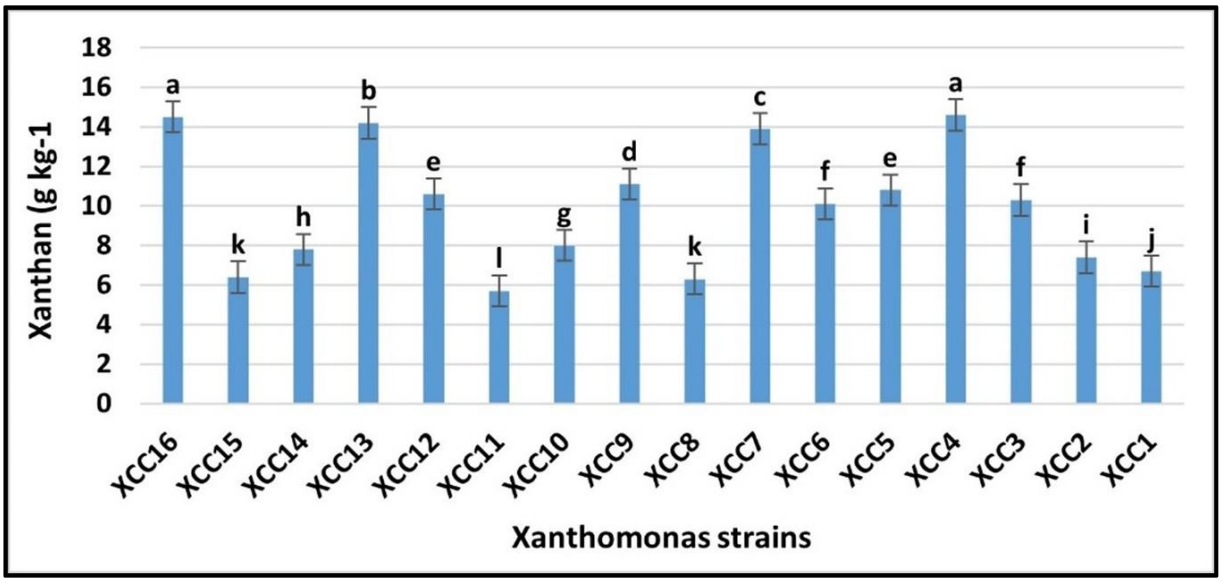

Figure (6): Xanthan gum production from sixteen strains of *Xanthomonas* (Means with the same letter are not significantly different).

An *et al*., (2020) recorded that some pathological characteristics of Xanthomonades could be correspond with xanthan output and pyruvic acid value. Recent results suggest that xanthan is in demand for pathogenicity and virulence, as we tested virulent strains (Which recorded strong disease symptoms on the leaves) which gave high xanthan amount *in vitro*. (Ramirez *et al*., 1988) recorded that virulence had nearly correspond with viscosity of *Xanthomonas* sp. bacteria. But, in the current study, the viscosities of gums were determined and gave high production in strains which gave low symptoms on leaves (low virulence) so, the results signalized very lower correlation between virulence and viscosity.

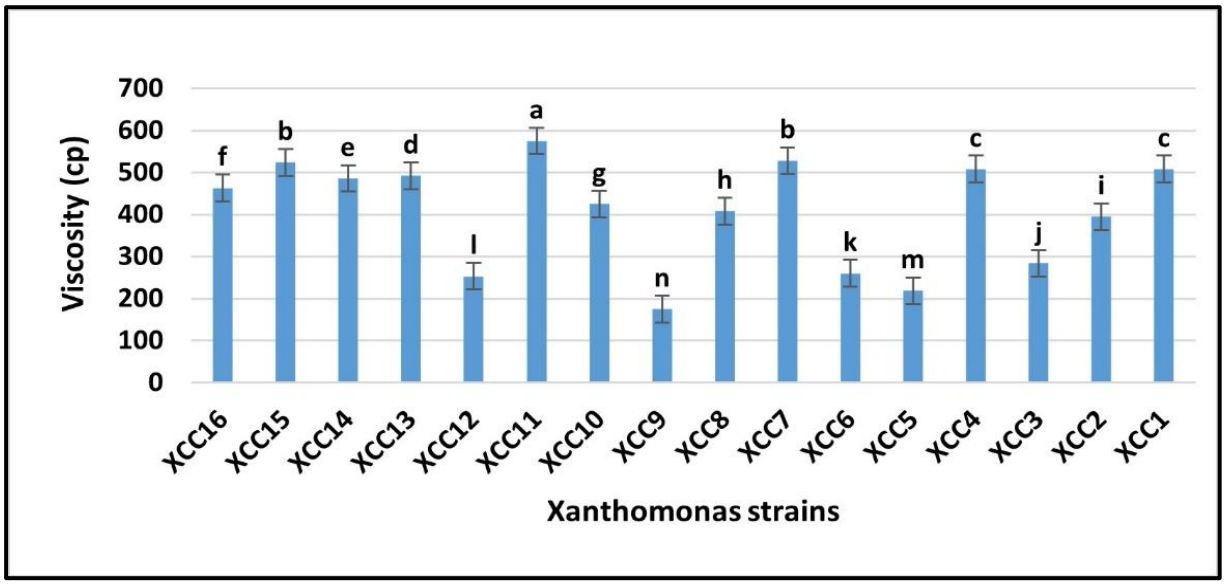

Figure (7): Viscosity determination from sixteen strains of *Xanthomonas* (Means with the same letter are not significantly different).

CONCLUSION

The virulence factors of *Xanthomonas campestris* pv. *campestris* isolates were examined. Through this work, it has been verified that there are many different virulence factors, which can be attributed to the survival and colonization of *Xanthomonas* microbes on the surface and inside the plant, and thus assessing the extent of its seriousness in causing the disease effectively and affecting plants, as it leads to economic losses in cabbage fields futurity as we showed that *X*. *campestris* pv. *campestris* has a battery of virulence factors typical for *Xanthomonas* sp., so it could be a cabbage pathogen. Data indicated that the increase in the production of lipopolysaccharides, adenosine kinase, cell adhesion, and the production of xanthan gum led to an increase in the virulence of *Xanthomonas* strains, while the viscosity of the strains is not considered one of the factors affecting disease severity or virulence.

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