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Original Article

In Vitro, Controlling the Establishment *of Xanthomonas Campestris* with different Bacterial Bioagents

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The antimicrobial agents of bacteria isolated from different rhizosphere of fruits and vegetables soil in Lahore. Of ten species, five were gram-negative (*Escherichia coli, Pseudomonas fluorescence, Klebsiella pneumoniae, Salmonella typhii, Brachybacterium faecium*); other five were gram positive and identified as *Bacillus farraginis, Kurthia gibsonii, Aureobacterium liquefaciens, Curtobacterium albidum, Micrococcus lylae*. The antagonistic potential of bacterial strains was assessed by the well diffusion technique and results indicating varying degree of biocontrol activity against pathogenic strain of *X. campestris*. Out of ten bacterial species, *E. coli* (gram negative) and *C. albidum* (gram positive) showed a high prevalence of resistance with reduction of 4.2cm and 4.1cm zone diameter respectively. The minimum inhibitory volume (MIV) to two bio-agents was determined for *X. campestris* from range 10-100 µL. *E. coli* (volume required to inhibit < 20 µL) and *C. albidum* (volume required to inhibit < 40 µL) exhibited good activity against pathogen. These results provide information on the prevalence of resistant bacterial strains with the MIV of organisms and indicate the possibility of using these bacterial species as bio-agent against *X. campestris*.

Key words: *Xanthomonas campestris*, gram positive bacteria, gram negative bacteria

Introduction

The genus *Xanthomonas* is a diverse and economically essential group of bacterial phytopathogens, belonging to the gamma-subdivision of the Proteobacteria. *Xanthomonas campestris* is the most important member that causes a variety of plant diseases[1]. *X. campestris* causes different diseases in plant foliage by producing black rot, canker, leaf spot and blights. These diseases may destroy leaves, petioles and stems rendering infected plants unsightly and unsalable[2,3].

The use of chemical compounds has failed to control plant diseases due to resistance, environment pollution, and damage to human health. Because of these disadvantages, the use of microorganisms for pathogen control and for plant growth promotion is becoming more common[4]. However, the success of biocontrol and yield increase depends on the nature of the antagonistic properties and on the mechanisms of action of the organism. The modes of action are widely varied and can be, for instance, nutrient competition, direct parasitism, and production of secondary metabolites[5]. Biological control of plant pathogens using antagonistic bacteria is a promising strategy for plant protection[6].

Bacillus species, gram-positive bacteria, are good biological control agents (BCA) for, their ability to produce different types of antimicrobial compounds, such as antibiotics (e.g., bacilysin, iturin, mycosubtilin), siderophores and to induce growth and defense responses in the host plant[7,8]. However, gram-negative bacteria belonging to *Pseudomonas* genera significant attention for antagonistic activity[7-11]. The aim of this work was to evaluate biocontrol potential of different gram positive and gram negative bacterial species against pathogenic strain of *Xanthomonas campestris*.

Materials and Methods

Isolation of bacterial bioagents

Soil samples were randomly collected from four different sites near Lahore, Pakistan, in sterilized plastic bags until (Table 1). The samples were processed using the soil dilution plate[12]. For soil dilution, one gram of soil diluted in 10ml of sterilized distilled water, course partials were removed by filtration through a layer of gauze. One ml of filtrate was used to make serial dilution of soil samples up to 10^5. For bacterial isolation, 1ml of 10^5 dilutions was added on solidified Luria Bertani (L.B) agar medium (g/L) plates. The dilution was spread with sterilized spreader and the plates were placed in an incubator at 37°C for 24 hours. Distinct individual colonies purified by streaking on a new nutrient agar plate. Pure cultures were identified according to the literature[13]. Selected bacterial species were: gram positive (*Bacillus farraginis, Kurthia gibsonii, Aureobacterium liquefaciens Curtobacterium albidum, Micrococcus lylae*) and gram negative (*Escherichia coli,*

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Pseudomonas fluorescence Klebsiella pneumoniae, Salmonella typhii, Brachybacterium faecium).

Pathogen

A bacterial strain, *Xanthomonas compestris* (FCBP 001) used for this study was obtained from First fungal Culture Bank of Pakistan (FCBP), University of the Punjab Lahore (Table 1). Cultures were revived on Luria Bertani (L.B) agar media at 37±2 ºC and used for further studies.

Table 1: *List of bacterial bioagent*

Name of bioagent	Source	Location
Gram Positive Bacteria		
Bacillus farraginis	Wheat field soil	Lahore
Kurthia gibsonii	Vegetable field soil	Lahore
Aureobacterium liquefaciens	Vegetable field soil	Lahore
Curtobacterium albidum	Gram field soil	Lahore
Micrococcus lylae	Vegetable field soil	Lahore
Gram Negative Bacteria		
Escherichia coli	Mango field soil	Lahore
Pseudomonas fluorescence	Vegetable field soil	Lahore
Klebsiella pneumonia	Sugarcane field soil	Lahore
Salmonella typhii	Sugarcane field soil	Lahore
Brachybacterium faecium	Vegetable field soil	Lahore

Preparation of the bacterial suspensions

The antagonistic and pathogenic strains were grown on Luria Bertani (L.B) agar media plates separately, incubated at 37±2 ºC for 24 h. Inocula of each strain were prepared by adding 5mL of a sterile saline solution (0.85% NaCl) to the Petri dishes. The cultures were scraped with a glass rod and the suspensions homogenized by agitation in a Vortex mixer. The amount of inoculum was measured in a spectrophotometer and adjusted with sterile saline solution (OD600 = 0.1 was equivalent to 1×10^8 colony forming units (CFU)/ml)[14].

Antimicrobial bioassays

A bacterial suspension for inocula and bioagents from 24h old culture were used by well diffusion method. Petri dishes (90 mm) containing Luria Bertani (L.B) agar medium were surface inoculated with 0.08 ml of bacterial inocula. After 15 min inoculation, one well of 8mm diameter was dug out in the agar medium, filled with 0.07 ml of bioagent suspensions. After 24h incubation at 37°C, the antibacterial effect was determined by measurement of the inhibition zone diameters.

Determination of Minimum Inhibitory Volume

The maximum inhibition diameter of above gram positive and negative bacterial species was checked again by minimum inhibitory volume. The bioagent suspensions were loaded into sterile well on L.B agar medium in different volumes of 10µL, 20µL, 30µL, 40µL, 50µL, 60µL, 70µL, 80µL, 90µL and 100µL concentration respectively and allowed to incubated for 24 hours at 37°C, the minimum inhibitory volume effect was determined by measurement of the inhibition zone diameters.

Statistical evaluation

The antimicrobial activity was determined by measuring the diameter of zone of inhibition that is the mean of triplicates ± SE of three replicates.

Results

Screening of gram positive bacterial strains

Five gram positive bacterial species viz. *B. farraginis, K. gibsonii A. liquefaciens, C. albidum* and *M. lylae* were screened for their antagonistic activity against *X. campestris*. Experimental results showed that all tested bacterial species show varying degree of biocontrol potential against *X. campestris* (Fig. 1). *C. albidum* showed effective biocontrol potential with 4.1cm diameter of inhibition zone. While in case of *K. gibsonii* and *M. lylae*, zone diameters were effectively reduce upto 2.2cm. On the other hand *B. farraginis* and *A. liquefaciens* were moderately effective and reduced the pathogenic colony with 3.1cm and 3.0cm zone diameter respectively.

Selection of gram negative bacterial strains

Five gram negative bacterial species viz. *E. coli, P. fluorescence, K. pneumoniae, S. typhii* and *B. faecium* were screened for their antagonistic activity (Fig. 1). *E. coli* showed most effective biocontrol potential with 4.2cm whereas *P. fluorescence* was weak to least effective to control the growth of *X. campestris*. While in case of *K. pneumoniae* and *B. faecium*, were effectively reduced the zone upto 2.4cm and 2.3cm diameters, respectively. Although, *S. typhii* was moderately effective against the pathogenic species with 3.0cm zone diameter.

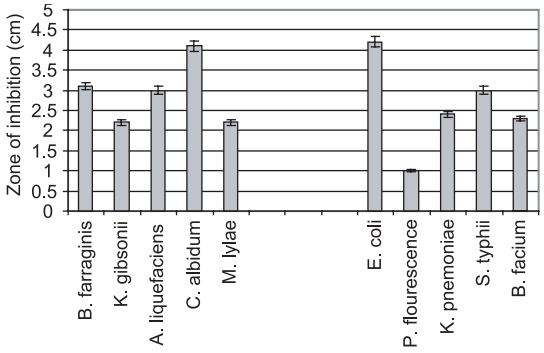

Fig 1: *Diameter of Inhibition zone of Gram positive and Gram negative bacteria*

Comparison of gram positive and gram negative bacteria antagonism with Minimum Inhibitory volume

The volume of antagonistic bacteria fell in the range of 10µL to 100µL for *C. albidum* (gram positive) and *E. coli* (gram negative) (Table 2). Table 2 also shows that the gram negative species of *E. coli* detected the most potent inhibition for *X. campestris* as compared to gram positive species. The minimum inhibitory volume of *C. albidum* that completely stopped the growth of *X. campestris* was < 40µL and it's above. On the other hand, only volumes of < 20µL completely inhibit the growth of pathogen in case of *E. coli*.

Table 2: *Minimum inhibitory volume for gram positive and gram negative bacterial species against X. campestris*

Minimum Inhibitory volume	Diameter of Inhibition Zone (cm)	
	Curtobacterium albidum (gram +ve)	*Escherichia coli* (gram -ve)
10 µL	na	na
20 µL	na	na
30 µL	na	0.43±0.03
40 µL	na	1.20±0.16
50 µL	1.90±0.03	2.10±0.03
60 µL	2.10±0.13	3.00±0.03
70 µL	2.90±0.06	3.60±0.10
80 µL	3.70±0.03	4.00±0.03
90 µL	3.90±0.03	4.10±0.03
100 µL	4.00±0.03	4.40±0.03

na: pathogen not active at volume range

Discussion

The results indicated that *X. campestris* showed antibacterial activities towards the Gram-positive and Gram negative bacteria. These results are consistent with previous reports on related food borne fungi regarding Gram-positive and Gram negative bacteria[15]. The resistance of Gram-negative bacteria to pathogen was not unexpected as; in general, this class of bacteria is more resistant than Gram-positive bacteria. Such resistance could be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism[16].

This study showed variation in antimicrobial potential among different soil bacterial isolates. All bacterial isolates exhibited antimicrobial activity against tested pathogen. The high proportion of antimicrobial producing strains may be associated with an ecological role, playing a defensive action to maintain their niche, or enabling the invasion of a strain into an established microbial community[17].

Results indicated that exhibited maximum inhibitory activity whereas *K. gibsonii* and *M. lylae* was less effective as in case of gram positive bacteria. Although different studies reported the antimicrobial potential of *C. albidum* against pathogenic fungi, like *Alternaria cajani*, *Curvularia lunata*, *Fusarium sp.*, *Bipolaris sp.* and *Helminthosporium sp.*[18]. Results also showed that *B. farraginis* and *A. liquefaciens* were moderately effective and exhibited almost similar biocontrol potential against *X. campestris*. Previously, antifungal potential of *Bacillus* sp, *Pseudomonas* sp. and *Escherichia* sp. has also been reported to inhibit the mycelial growth of many species of *Aspergillus, Penicillium* and *Fusarium*[15,19,20].

In present study, *Escherichia coli* showed highly effective biocontrol prospective against *X. campestris* where it reduce the pathogenic growth with 4.2 cm zone diameter. But other studies have reported cytosolic proteins of *Escherichia coli* are responsible for antimicrobial potential against pathogenic strains[15,21,22]. Our results demonstrate that the growth of *X. campestris* was remarkably inhibited by the minimum inhibitory volume of E. coli (0.43 cm / < 30µL inhibition zone) and *C. albidum* (1.90 cm / < 50µL inhibition zone). It seems very likely, therefore, that the antibacterial compound from bioagents may inhibit pathogen by a different mechanism than that of currently used antibiotics and may have therapeutic value as an antibacterial agent against multi-drug resistant bacterial strains and must be better explored in future. The presented data exhibit the antimicrobial activity of bacterial species and indicate the possibility of using these bacterial species as a biological agent to control pathogenic species. However, biological agents tested in this study should be investigated extensively for food safety before commercialization.

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