IN VITRO SCREENING OF SOME BIOCONTROL AGENTS AGAINST *XANTHOMONAS AXONOPODIS* PV. *MALVACEARUM* ISOLATED FROM INFECTED COTTON PLANTS

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ABSTRACT

Cotton (Gossypium spp.) Is the most important fiber and cash crop in the sudan. Among the inflicting disease, bacterial angular leaf spot caused by Xanthomonas axonopodis pv. malvacearumm (Xam) it can cause serious damage under favorable conditions . Management of the disease by use of synthetic chemicals was discouraged recently due to their hazardous nature and pollution to the environment. In this study different concentrations of chitosan compound (1,3, 5 and 10 mg/ml), fourteen strains of antagonistic bacteria Paenibacillus, water extracts and oil of some plants (viz. Syzygium aromaticum, Nigella sativa,Trigonella foenum graecum and blubs of Allium sativum) and camel urine were screened for their efficacy in inhibiting the growth of Xanthomonas axonopodis pv. malvacearum in vitro. All concentrations of chitosan tested had inhibitory effects against the bacteria except 1mg/ml. Chitosan at 10 mg/ ml showed the largest inhibition zone of 14mm, followed by 5mg/ ml and 3mg/ ml which gave inhibition zones of 12mm and 11 mm, respectively compared to the control. Only two strains of the genus Paenibacillus had inhibitory effects against the bacteria, strain (4) and strain (8), which gave inhibition zones of 11mm and 8mm, respectively, compared to the control. Among all plant extracts, their oils and camel urine used in this study, only water extract of Syzygium aromaticum showed inhibitory effect against isolated bacterium and recorded inhibition zone of (6mm).

Key words: Biocotrol, *Xanthomonas*, Chitosan, Botanic extract & Cotton

Introduction

Cotton (*Gossypium* spp.) which belongs to family Malvaceae known as (White Gold). It is largely grown as an irrigated crop, and as rain fed in areas where rainfall satisfies its needs. It affected by various Fungal. Bacterial, and viral diseases. Angular leaf spot incited by bacteria *Xanthomonas axonopodis* pv. *malvacearumm* , is one of the most destructive diseases of cotton that causing severe qualitative and quantitative losses in most cotton-growing areas of the world (Abdo-Hasan *et al*, 2008). In Sudan the disease was first reported in 1922 by Tarr and reported to cause considerable crop losses and reduced cotton yield depending on the prevailing environmental conditions and developmental stage at which the infection took place (Mohammed *et al*., 2003).It affects yield and yield component directly or in directly as it causes seedling blight, severe leaf shedding boll shedding, boll rotting and lint staining. As there is no bactericide registered in Sudan to stop disease development so far; development of resistant varieties considered the only effective control measure. Breeding for resistance efforts has been hampered due to external economic and politic factors, as well as the ability of the pathogen to break-down plant resistance (Mohammed *et al*., 2003). These reasons incited the need for the development of alternative strategies for management of this disease. Biological control involving microbial agents or biochemical offers an eco-effective alternative as an important component of an integrated disease management program (Li *et al*., 2007). Chitosan is a natural polysaccharide derived by deacetylation of chitin, a major component of the shells of crustacean such as crab and shrimp, exoskeleton of insect and cell walls of some fungi. In recent years Chitosan has attracted attention because of its unique physical, chemical characteristics and biological activities including biodegradability, nontoxicity and antimicrobial activity. Due to these characteristics it's used in vast array of widely and different products and application ranging from pharmaceutical cosmetic product to food additives, water treatment and plant protection (Gavhane *et al*., 2013 and ElHadrami *et al*., 2010). Application of chitosan in environmental protection and agriculture include its use as a biocontrol agent for controlling plant disease (Khan *et al.*, 2006). (Yanli *et al.* 2012) investigated the antibacterial activity of two kinds of chitosan against twelve *Xanthomonas* strains recovered from *Euphorbia pulcherrima*,

indicated that both chitosan markedly inhibited bacterial growth based on Optical density loss. (Li *et al*, 2010) indicated the antibacterial activities of chitosan against *Pseudomonas aeruginosa*, *P. mirabilis* and *Escherichia coli* showed that chitosan could markedly inhibit bacterial growth (Algam *et al*, 2010) demonstrated that chitosan exhibited strong antibacterial activity against *Ralstonia sonlanacearum*. This study was carried out in attempt to investigate a new, eco-friendly and cheap alternative control agent(s) of natural origin that effectively controlling angular leaf spot of cotton, this because the causal agent possess ability to develop resistance against chemicals and/or produce races that severely infect resistant varieties (break-down of resistance). Moreover, a severe infection has been reported recently in some cotton-production areas (Personal communication, Agricultural Research Corporation, New Halfa and El-Rahad Stations). Therefore,this study aimed to Isolate and Identify the bacterial angular leaf spot from infected cotton plants and to screen the antibacterial activity of chitosan, some plant extracts, *Paenibacillus* spp. and camel urine against *Xanthomonas axonopodis* pv. *malvacearum* (Xam) that cause angular leaf spot in cotton plants *in vitro*. Herein we present promising results that suggest a potential application of the biocontrol agents studied as means of angular leaf spot control *in vitro*

Material And Method

Sampling

Sample of leaves exhibiting typical symptoms of bacterial angular leaf spot were collected from Faculty of Agriculture University of Khartoum for pathogen isolation. Samples were placed in plastic bags appropriately labeled and stored at 4 ºC for further analysis.

Isolation of the pathogen

Isolation of *Xanthomonas axonopodis* pv. *malvacearum* from collected samples was carried out at plant pathology laboratory, Department of Crop protection, Faculty of agriculture, University of Khartoum, Infected leaves were cut to small parts and then surface sterilized with 10 % sodium hypochlorite (NaOCl) for two min., thoroughly rinsed in sterilized distilled water several times to remove excess of sodium hypochlorite and soaked in sterile distilled water for 2 hrs. At room temperature to allow bacteria to disperse in to the surrounding liquid .The bacterium was isolated by serial dilution technique, o.1ml from each dilute was spread over the plates containing Nutrient Agar medium (NA) by using sterilized glass rod and incubated at 25-30°C for 5 days. Single colonies of bacteria were streaking onto the same medium and maintained on slant of nutrient agar and kept in the refrigerator for further use.

Identification of the pathogen

In order to identify the isolated bacteria different studies were conducted following the methods described by (Rafi *et al*., 2013), including Morphological, Cultural and biochemical tests.

Pathogenicity Test

To demonstrate the isolated bacterium is the causal agent of angular leaf spot on cotton plant, Pathogenicity test was performed on the same cotton cultivar from which the bacterium was isolated. The inoculum was prepared by streaking a loopful of isolate on the nutrient agar plates and incubated at 25-30°C for 48h. Then the bacteria harvested by scraping from the plate surface with sterilized distilled water(S.D.W).The concentration of bacterial cells was adjusted to an optical density of OD600=1.00 (approximately equal to$1.0x10^9$ CFU ml^{-1}) using a spectrophotometer, plants were artificially inoculated at the six true-leaf stage by injection the leaves on the lower surfaces into six inoculation points using syringe without needle and applying a constant pressure against the leaf until an area of mesophyll tissue water-soaked (Bielsa *et al.,* 2012).Plants were covered with plastic bags for 24 h to conserve moisture, whereas control was similarly inoculated with sterile distilled water(S.D.W.).

Preparation of chitosan

Chitosan extracted from shrimp shells (degree of N-deacetylation 75%; Sigma-Aldrich, U.S.A.) was solubilized in 1% acetic acid to obtain a concentration of 10 mg/mL (stock solution). The solution was alkalized to pH 5.6 with NaOH and autoclaved at 121°C for 15 min (Algam *et al*, 2010).

Collection of plant materials

Seeds of *Syzygium aromaticum*, *Nigella sativa*, *Trigonella foenum graecum* and blubs of *Allium sativum* were purchased from the local market, washed thoroughly 2 - 3 time with running tap water and then with sterile water followed by shade-dried, powdered and used for extraction.

Preparation of aqueous plant extracts

Samples (25g) of dried seeds of *Syzygium aromaticum, Nigella sativa, Trigonella foenum graecum* and blubs of *Allium sativum* were macerated with 100ml sterile distilled water in waring blender for 10 min. The macerate was filtered through Whatman No.1 filter paper and sterilized at 121°C for 15min. the extract was preserved aseptically in a bottle until further use. The extract was subjected to antibacterial activity assay (Babu *et al*., 2007).

Essential oils

Oil of plants *Syzygium aromaticum*, *Nigella sativa*, *Trigonella foenum graecum, and Allium sativum* were purchased from the local market and subjected to antibacterial activity assay.

Source of antagonistic bacteria *(Paenibacillus*)

Fourteen strains of *Paenibacillus* were obtained from the Plant Pathology laboratory Faculty of Agriculture University of Khartoum, and were maintained on NA medium.

Camel urine
Camel urine was obtained from Camel research center, University of Khartoum**.**

Antibacterial activity of chitosan
To determine the antibacterial activity of chitosan disk diffusion method on nutrient agar medium was performed (Cleci *et al.,* 2009). The inoculum of bacterial cells was adjusted to an optical density of OD600=1.00 (approximately equal to10^9 CFU/ml) using a spectrophotometer, and spread on the plates with a sterile cotton swab moistened with the bacterial suspension. Ten mm diameter sterilized filter paper disks (Whatman No1) were impregnated with different chitosan concentrations of 1, 3,5 and 10mg/mL and then placed on each plate, four disks per plate. Disks impregnated with sterilized distilled water were used as control. The plates were incubated at 28°C for 48h and possible inhibition zone was observed after 2 days, each treatment had four replicates.

Antibacterial activity of aqueous plant extracts and their oils
To evaluate the Antibacterial activity of some plant aqueous extract and their oils agar well diffusion Method was used (Amit and Singh, 2011) on nutrient agar medium. The inoculum of bacterial cells prepared as mentioned above using a spectrophotometer. The inoculum was spread on the solid plates with a sterile cotton swab moistened with the bacterial suspension. Four wells, 6mm in diameter on each plate was fill with the volume (50µl) of water extract of plants and oils. Controls were done with sterilized distilled water in the same way. The plates were incubated at 28°C for 48 h and possible inhibition zone was observed after 2 days, each treatment had four replicates.

Antibacterial activity of antagonistic bacteria

The antagonistic activity of fourteen *Paenibacillus* strains against *Xanthomonas axonopodis pv.malvacearum* was evaluated using method described by (Algam *et al*, 2010). Inoculum of bacterial cells adjusted as mentioned above using a spectrophotometer was spread on the solid plates with a sterile cotton swab moistened with the bacterial suspension; then sterile tooth picks were used to transfer the test strains of *Paenibacillus* from 2day-old culture. Four plates were used for each strain tested and four test spots were placed on each plate, the plates were incubated at 28°C for 48 h and possible inhibition zone was observed after 2 days.

Antibacterial activity of camel urine
Antibacterial activity of camel urine determined by disk diffusion method as mentioned above.

Statistical analysis
The experiments were laid out as completely randomized design. Collected data were subjected to the analysis of variance (ANOVA) and means were separated using Duncan's Multiple Range Test at the 5% level significance. All statistical analysis were performed by SPSS programme version 16.0

Results

Isolation of the pathogen from infected leaves
The bacterium that isolated from Cotton leaves was designated as *Xanthomonas axonopodis* pv *malvacearum* which showed typical symptoms of the disease. Purified culture of this isolate was obtained from single colony of bacterial growth in nutrient agar, and then maintained in nutrient agar slant.

Identification of the pathogen
Table (1) summarizes the cultural and biochemical characteristics of the isolate of bacterium on different culture media and their reaction in several key tests.

Pathogenicity test
The isolate was pathogenic on cotton leaves on which typical symptoms of bacteria were produced, the inoculated leaves of cotton appearing as water-soaked spot followed by chlorosis and necrosis and no spots were observed in control leaves of cotton inoculated with sterilized water.

Antibacterial activity of chitosan
The antibacterial activity of chitosan was evaluated *in vitro* against *Xanthomonas axonopodis* pv. *malvacearum* (Xam*)*. The growth of *Xanthomonas axonopodis* pv. *malvacearum* was significantly inhibited by three chitosan concentrations (3, 5 and 10 mg/mL) (P<0.05) compared to the chitosan concentration (1 mg/mL) and control, It was observed that the inhibitory effect increased as chitosan concentration increased. Diameters of inhibition zones ranged between 11-14 mm, the highest zone of inhibition was found at concentration of 10mg/ml. It was found that there were no significant differences between the 3 mg/ml and 5mg/ml of chitosan concentrations, but they were both significantly different from the concentration 10mg/ml.

The results given in Table (2) demonstrated observable reduction in the pathogen's growth in terms of inhibition zones. Which indicated that chitosan could markedly inhibit bacterial growth.

Antibacterial activity of plant extracts

The antibacterial properties of the aqueous extract and oils of some plants (viz. *Syzygium aromaticum, Nigella sativa, Trigonella foenum graecum* and blubs of *Allium sativum*) were tested against *Xanthomonas axonopods* pv.*malvacearum*. Table (3) shows the results.

In this study, all plant extracts (water extract and oils) tested didn't affect bacterial growth except the water extract of *Syzygium aromaticum* which gave inhibition zone of (6mm).

Antibacterial activity of antagonistic bacteria
Two out of 14 *Paenibacillus* strains showed inhibitory effects *in vitro* on the growth of Xam. The inhibition zone of strain 4 was (11mm) while that strain 8 was (8mm). None of the other 12 *Paenibacillus* strains reduced the growth of bacteria Table (4).

Antibacterial activity of camel urine
The bacterial isolate was found to be resistant against camle urine.

Table 1: Cultural and Biochemical characteristics of the Bacterium

Characteristic	Result
Growth on common media (NA)	+
Gram stain	−
Anaerobic growth Test	+
Motility	+
Growth at 40°C	−
Colony mucoid and yellow on YDC medium	+
Starch hydrolysis	+
Oxidase Test	−
Catalase Test	+

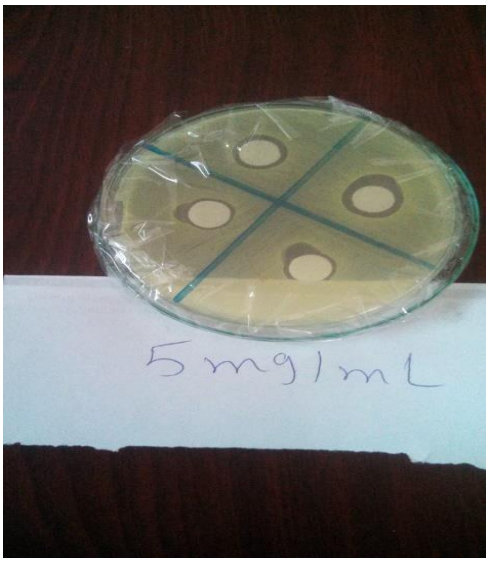
+ = Positive − = Negative

Table 2: The inhibitory effect of different concentrations of chitosan on *Xanthomonas axonopodis* pv. *malvacearum* growth.

Concentration of chitosan (mg/mL)	Diameter of the zone of inhibition(mm)
10	14.38[a]
5	12.75[b]
3	11.68[b]
1	00.00[c]
Control	00.00[c]

Means followed by same letters were not significantly different at $P < 0.05$.

Effects of different concentrations of chitosan on the growth of *Xanthomonas axonopodis* pv. *malvacearum*



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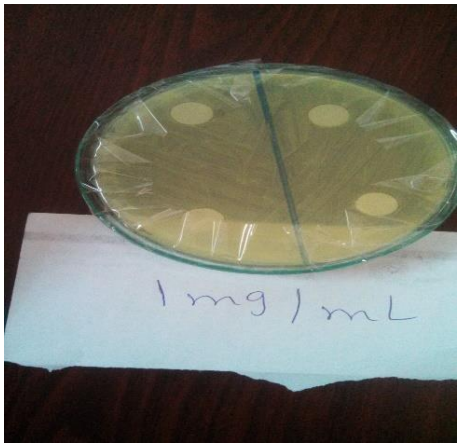


Table 3: Zone of inhibitory activity (in millimeter) of some plant extracts (water extract and essential Oils) against *Xanthomonas axonopodis* pv. *malvacearum* growth

Plant species	Water extract	Essential Oils

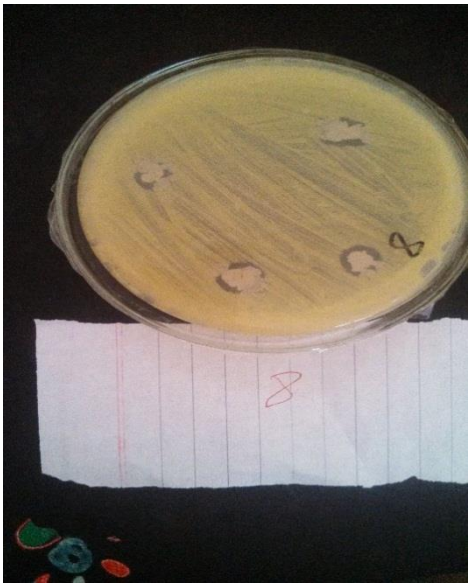
Syzygium aromaticum	06:59	00.00
Trigonella foenum	00.00	00.00
Nigella sativa	00.00	00.00
Allium sativum	00.00	00.00
Control	00.00	00.00

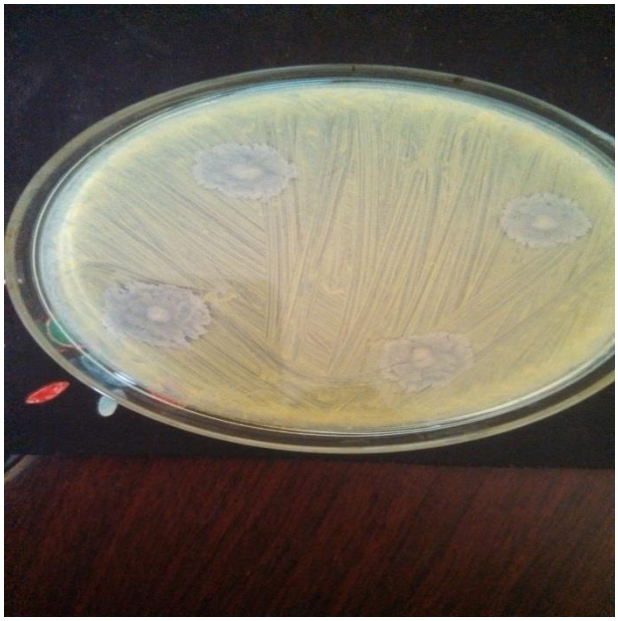
Table 4: The inhibitory effect of *Peanibacillus* strains on *Xanthomonas axonopodis* pv. *malvacearum* growth.

***Peanibacillus* strains**	**Diameter of the zone of inhibition(mm)**
1	00.00^{c}
2	00.00^{c}
3	00.00^{c}
4	11.00^{a}
5	00.00^{c}
6	00.00^{c}
7	00.00^{c}
8	08.50^{b}
9	00.00^{c}
10	00.00^{c}
11	00.00^{c}
12	00.00^{c}
13	00.00^{c}
14	00.00^{c}
Control	00.00^{c}

Means followed by same letters are not significantly different ($P< 0.05$).

(Fig. 2): The inhibitory effect of *Peanibacillus* strains (4 and 8) on *Xanthomonas axonopodis* pv. *malvacearum* growth.





Discussion

The presence of angular leaf spot disease was reported previously in cotton and it is one of the economically important diseases in cotton production. It is affects all plant parts and under humid conditions it leads to cause considerable crop losses (Mohammed *et al*, 2003).

The present study included isolation of bacteria from infected cotton leaves, the isolate formed yellow, convex circular and mucoid colonies, rod shape, motile cells were Gram negative, obligate aerobes, catalase positive, oxidase negative and exhibited strong starch hydrolysis agreed with (Saeedi *et al*, 2010; Rafi *et al*, 2013; Mohammed, 2011; Opara and Odibo, 2009).

In the present study chitosan concentrations (1, 3, 5 and 10 mg/mL), were screened *in-vitro* against the *Xanthomonas axonopodis pv. malvacearum*, where application of chitosan gives promising results, expressed as strong antibacterial activity against *Xanthomonas axonopodis pv. malvacearum*. These results were agreed with the results of (Li *et al 2008*, 2010; Algam *et al.*, 2010) who reported that chitosan could inhibit the growth of *Xanthomonas axonopodis* pv. *poinsettiicola* strains and *Ralstonia solanacearum* under different environments.

Several studies have demonstrated that the antibacterial effect of chitosan could be attributed to the interactions between positively charged chitosan molecules and negatively charged residues on the bacterial cell surface of Gram-negative bacteria (Helander *et al*., 2001). Furthermore, (Chung and Chen, 2008) found that the inactivation of *Escherichia coli* by chitosan occurs

via a two-step sequential mechanism: initial separation of the cell wall from its cell membrane, followed by destruction of the cell membrane.

Only two strains of antagonistic bacteria of the fourteen Paenibacillus strains examined, inhibited the growth of *Xanthomonas axonopodis pv. malvacearum.* This is partially consistent with the result of (Li *et al.*2007; Algam *et al*, 2010) who found that the *in vitro* growth of *Ralstonia solanacearum* and *Pythium* was unaffected by the same strain of *Paenibacillus*.

some plants extracts (viz. *Syzygium aromaticum, Nigella sativa, Trigonella foenum graecum* and blubs of *Allium sativum*screened in this study didn't express any antibacterial activity except water extract of *Syzygium aromaticum*, this results which is in agreement with the results of (Sulieman *et al.*, 2007; Ram *et al.*, 2010; Masoud and Gouda, 2012) who demonstrated that the clove extract exhibited the maximum zone of inhibition against various pathogenic bacteria.

Camel urine did not inhibit the growth of *Xanthomonas axonopodis pv. malvacearum.* This result agreed with (Munir, 2011) who reported that no inhibition activity observed after 24 hours of incubation; but after 48 hours a very slightly inhibited zones were observed in *Staphylococcus aureus* and *Streptococcus*.

Conclusion And Recommendations

According to these results, it could be suggest that Chitosan is a potential mean for use in control of *Xanthomonas axonopodis pv. malvacearum.* It is markedly inhibited the growth of the bacteria and its activity was higher than that of the *paenibacillus* strains 4 and 8; and water extract of *Syzygium aromaticum.* From these results and finding of other workers, it could concluded that chitosan offers a safe alternative to synthetic pesticides and considered as a potential agrochemical of low environmental impact. Further investigations must be carried out to evaluate their efficacy for control the angular leaf spot cause by *Xanthomonas axonopodis pv. Malvacearum in vivo.*

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