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Original Research

Use of Botanicals Against Citrus Canker (*Xanthomonas axonopodis* pv. *citri*) Affecting Lime (*Citrus aurantifolia* Swingle) in Sudan

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ABSTRACT

Aqueous extracts (2%) of three plants; roselle (*Hibiscus subdariffa* Linn.), cinnamon (*Cinnamomum verum* J. Presl.) and clove (*Syzygium aromaticum* Linn.) were assessed for control of citrus canker disease in lime (*Citrus aurantifolia* Swingle) caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) under *in vitro* and *in vivo* conditions. Mancozeb fungicide (1.5%) was used as a positive control. In the first experiment, the effect of these three plant extracts was investigated *in vitro* through inhibition zone technique. Results indicated that roselle and cinnamon extracts caused significantly less inhibitory effect (38.5% and 25%, respectively) on canker severity compared to the fungicide Mancozeb (control), but they were significantly ($P \geq 0.05$) more efficacious than clove extract. Clove extract showed little or no *in vitro* inhibitory activity. In the second experiment conducted in a greenhouse with natural infection of *Xac* on lime seedlings, aqueous extracts (2%) of roselle, cinnamon and clove significantly ($P \geq 0.05$) exhibited an inhibitory effect against the disease severity with a decline from 40.4 to 45.2%. Roselle extract was the most effective for control of citrus canker (45.2%) followed by cinnamon and clove which recorded only 40.4% disease control after three weeks compared to the control treatment.

Keywords: *Antimicrobial effect, bacterial disease, bio-pesticides, botanical extracts, disc diffusion technique, inhibition zone.*

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INTRODUCTION

Citrus canker disease (CCD) caused by *Xanthomonas axonopodis* pv. *citri* is the most devastating economically important bacterial disease prevailing in citrus growing regions. Several citrus species including grapefruits, limes, sour oranges, lemons, and oranges are highly susceptible to this disease (Islam et al., 2014; Islam et al., 2019; Martins et al., 2020). The disease is believed to have originated in South East Asia but presently it is also prevalent in Africa, Asia, and Australia. The disease is extremely persistent when it becomes established in an area (Khuntong et al., 2014; Negi and Kumar, 2015; Chethankumar et al., 2017). It is identified as a major threat affecting leaves, twigs, petioles, branches, and fruits that causes considerable damage both quantitatively and qualitatively. In addition, it has a negative impact on the international trade (Graham et al., 2004).

The causal bacterium *Xanthomonas axonopodis* pv. *citri* has different forms (A, B and C) depending on their host range and their geographical distribution (El-Goorani, 1989; Alizadeh and Rahimian, 1990; Traore et al., 2008; Derso et al., 2009; Juhasz et al., 2013; Al-Saleh et al., 2014). In Sudan, typical disease symptoms were observed for the first time in Gadaref State (near the Sudanese-Ethiopian border) throughout the fall season of 2013, only on lime (*Citrus aurantifolia* Swingle), but no symptomatic trees were encountered in canker susceptible grapefruit and orange varieties grown in the same orchards (Elhassan et al., 2014; Abubaker et al., 2016). Typical symptoms of CCD were produced naturally on lime trees only consisting of erumpent corky lesions surrounded by water-soaked tissues and a yellow halo on leaves, fruits, and branches. In serious infections, fruit lesions with crater-like centers, frequent fruit drops, extensive defoliation, and dieback of twigs and branches were generally evident. CCD usually occurs on seedlings and on young and adult trees of susceptible hosts in which there is a flush of actively growing shoots and leaves from late summer through fall in most citrus growing areas (Elhassan et al., 2014; Abubaker et al., 2016). Wounds from wind, thorns, insects, and mechanical damage contribute to the infection of mature tissues. CCD causes significant losses when infection occurs at an early stage of plant growth. One major limitation of using chemical control agents is that phytopathogenic bacteria frequently develop a resistance to these compounds (Islam et al., 2014). In recent years, great interest has been shown in the antimicrobial effects of certain plant extracts for the control of plant diseases. These plant extracts have been reported as effective inhibitors of phytopathogenic bacterial growth and at the same time avoid or reduce the harmful effects of synthetic pesticides on the

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ecosystem or the environment (Leksomboon et al, 2000; Mahajan and Das, 2003). Plant extracts provide another source of natural pesticides to control plant pests (Leksomboon et al., 2001). Therefore, the goal of this study was to assess the potential control of lime canker using plant extracts under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Isolation and purification of *Xanthomonas axonopodis* pv. *citri*

Isolation and purification of the bacteria from infected leaves, fruits and twigs was conducted following the National Diagnostic Protocol for Asiatic Citrus Canker (NDP, 2014). The bacteria were grown on nutrient agar.

Inoculum preparation

Pure isolates of the bacterium were grown on nutrient agar plates and incubated at 28 °C for 24 h. Bacterial cells were then harvested in sterile distilled water by using sterile glass rod and the bacterial suspension was adjusted finally to give 1.0×10^8 CFU/mL using a UV spectrophotometer at a wavelength of 600 nm (Sunrise Spectrophotometer, Tecan).

Preparation of aqueous plant extracts

Three plants, roselle (*Hibiscus subdariffa* Linn.), cinnamon (*Cinnamomum verum* J. Presl.) and clove (*Syzygium aromaticum* Linn.) were used in this study. The information regarding these plants and their parts used for preparation of aqueous extracts to control the bacterium are presented in Table 1.

Table 1: Plant extracts used for the control of *Xanthomonas axonopodis* pv. *Citri.*

Common name	Botanical name	Family	Plant parts used
Roselle	*Hibiscus subdariffa* Linn.	*Malvaceae*	Dry flower
Cinnamon	*Cinnamomum verum* J. Presl.	*Lauraceae*	Inner bark
Clove	*Syzygium aromaticum* Linn.	*Myrtaceae*	Flower bud

Five grams of dried material of each plant species were thoroughly washed under tap water and shade dried. Later, placed in a 250 mL conical flask and then 250 mL of sterile distilled water was added to each flask. The mixture was placed on a Shaker (Orbital incubator S150) and left to extract for 18 h at a speed of 133 rpm at room temperature (25 °C). Extracts were passed through two layers of cheesecloth and the filtrates were then collected in 50 mL round bottom flasks and their antibacterial activity against the citrus canker bacterium was tested at a concentration of 2% under *in vitro* and *in vivo* conditions. Mancozeb (Dithiocarbamate) fungicide was also used in this study as a positive control at a rate of 1.5%.

In vitro experiment

The effectiveness of these plant extracts was tested by disc diffusion technique (Negi and Kumar, 2015). 100 µl of bacterial suspension (1.0×10^8 CFU/mL) were spread onto the surface of nutrient agar plate using sterile cotton swabs. Sterile filter paper discs (6 mm) were dipped briefly in the respective plant extracts and were then applied on to the surface of the inoculated nutrient agar plates. Discs impregnated in Mancozeb were used as positive controls, while sterile distilled water-treated discs were used as a negative control. The treated plates were incubated at 28 °C in an incubator for 48 h and the developing inhibition zones were observed and measured to determine the relative efficacy of each plant extract against the bacterium. The recorded data were subjected to analysis of variance (ANOVA) according to the method described by Gomez and Gomez (1984) for a completely randomized design, applying EXCEL computer package version 2010. The least significant difference (LSD) test was used for the separation of treatment means.

In vivo experiment

The investigation was conducted on one-year-old lime seedlings that were kept in greenhouse conditions at a temperature of 25-30 °C. Eighty healthy lime seedlings were divided into four batches (A, B, C and D), each batch contained 20 transplants. Infected lime seedlings with citrus canker were distributed randomly between and around the treated plants. Water splashes were also created every day to make them more vulnerable to bacterial infection. Batch A was sprayed by roselle, and batch B and C by cinnamon and clove extracts, respectively, while batch D was not sprayed (control). All batches were sprayed at a rate of 2% concentration. The spraying process was repeated at every 7 days interval for 3 weeks using fresh plant extracts every time. The recorded data on the disease severity were subjected to analysis of variance (ANOVA) following the method described by Gomez and Gomez (1984) for a completely randomized design, employing EXCEL computer package version 2010. The least significant difference (LSD) test was used for the separation of treatment means.

RESULTS

Antibacterial activity of plant extracts against the bacterium *in vitro*

To assess the efficacy of selected plant extracts (roselle, cinnamon and clove) against *Xanthomonas axonopodis* pv. *citri in vitro*, an experiment was conducted, and the evaluation was made by disc diffusion method (Fig. 1). Antibacterial activity in selected plant extracts was tested. Significant differences ($P \leq 0.05$) were obtained between the tested treatments. The data presented in Table 2 revealed the significant differences in zones of inhibition (in mm). The roselle extract was found significantly superior in controlling the growth of the bacterium producing maximum zones of inhibition (9.25 mm), followed by cinnamon extract (6.50 mm), while clove extract demonstrated no inhibitive effect against *Xanthomonas axonopodis* pv. *citri*. In comparison, the antibacterial activity of roselle and cinnamon recorded 38.5% and 27.1%, respectively, relative to Mancozeb.

Assessment of plant extracts under greenhouse conditions

In the greenhouse experiment, extracts from roselle, cinnamon

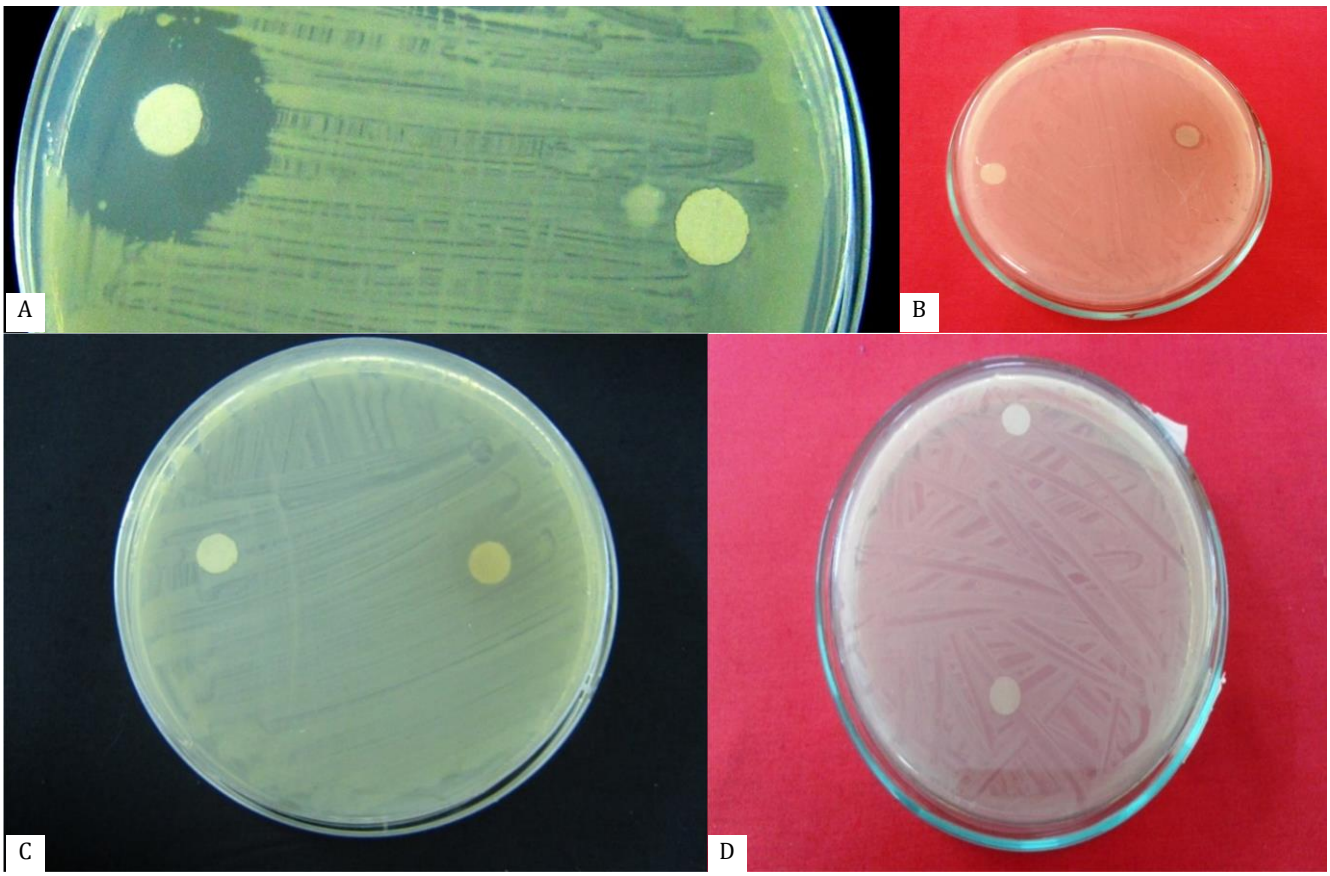

Figure 1: Inhibition zone (mm) of (B) Roselle aqueous extracts (9.25 mm), (C) Cinnamon (6.5 mm) and (D) Clove (0.0 mm) on *Xanthomonas axonopodis* subsp*. citri* multiplication in comparison with (A) Mancozeb (1.5%) used as a +ve control (24.0 mm) and Distilled water used as a -ve control (0.0 mm).

and clove significantly reduced the canker disease severity when sprayed over naturally infected lime seedlings particularly at 3-week intervals as compared to control (Table 3). Roselle extract was the most effective to control the citrus canker in which the disease declined by 45.2%, followed by cinnamon and clove which recorded only 40.4% disease decline.

DISCUSSION

Bacterial canker of citrus caused by *Xanthomonas axonopodis* pv. *citri* is a newly emerging and most devastating disease naturally infecting lime (*C. aurantifolia* Swingle) trees in most of the states of Sudan. For the last 10 years, the plant extracts have been used worldwide in the human and animal diseases therapy (Vudhivanich, 2003). There are many reports on use of plant extracts in plant diseases control. However, only few reports are available on phytopathogenic bacteria. Furthermore, the advent of increased antibiotic resistance, this bacterium is worsening the situation and stressing to research other alternatives for its control (Islam et al., 2014). This study was designed to investigate the efficacy of three plant extracts for the effective control of citrus canker on lime plants both under laboratory and greenhouse conditions.

Under laboratory conditions (*in vitro*), roselle (*Hibiscus subdariffa*) demonstrated higher inhibitory effects with inhibition zones of 9.25 (38.5% compared to Mancozeb). The inhibitory level obtained for roselle could be enhanced if the floral parts used for the experiments are fresh and the concentration dose of the aqueous extract is increased. The effectiveness of *Hibiscus subdariffa* was previously reported by many investigators. Leksomboon et al. (2001) demonstrated that the spray with the leaf extract of *Hibiscus subdariffa* could reduce the incidence of citrus canker to 78% compared to 100% in control plants. Similarly, in an *in vitro* experiment, Mohammed

Table 2: *In vitro* efficacy of selected plant extracts against *Xanthomonas axonopodis* pv. *Citri.*

Treatments	inhibition zone (mm)	Inhibition (%) relative to Mancozeb (control)
Mancozeb at 1.5% (+ control)	24.00 a	100.00
Roselle aqueous extract (2%)	9.25 b	38.54
Cinnamon aqueous extract (2%)	6.50 c	27.08
Clove aqueous extract (2%)	0.00 d	00.00
Distilled Water (- control)	0.00 d	00.00

Means followed by same letter(s) are not statistically different at $P \leq 0.05$. LSD for treatments at 5% = 0.937.

Table 3: *In vivo* efficacy of selected plant extracts against *Xanthomonas axonopodis* pv. *Citri.*

Plant extract	Disease severity (Proportion of disease decline in %)		
	Week 1	Week 2	Week 3
Roselle	0.60 a (66.6)	1.20 a (58.6)	2.30 a (45.2)
Cinnamon	0.90 a (50.0)	1.80 b (37.3)	2.50 a (40.4)
Clove	1.00 a (44.4)	1.50 ab (48.2)	2.50 a (40.4)
Control (untreated)	1.80 b (0.00)	2.90 c (0.00)	4.20 b (0.00)

LSD for treatments at 5% = 0.41 (for week 1), 0.42 (week 2), 0.55 (week 3). Values between parentheses are representing the proportion of diseases decline (%).

(2019) demonstrated that *Hibiscus subdariffa* (roselle) and *Cinnamomum verum* (cinnamon) recorded significantly higher inhibitory effects with inhibition zones of 3.15 and 3.15%, respectively, when were used at high concentrations (25 and 12.5%) compared to the positive control (Amoxicillin 4%) which recorded an inhibition zone of 5.33%.

Investigation on the efficacy of plant extracts for effective control of citrus canker was also conducted on naturally infected lime seedlings under greenhouse conditions. Aqueous extracts of *Hibiscus subdariffa*, *Cinnamomum verum* and *Syzygium aromaticum* significantly reduced the canker disease severity when sprayed over naturally infected lime seedlings particularly at 3-week intervals as compared to control. Roselle, cinnamon and clove showed reductions in disease severity in comparison to the control by 45.2, 40.4 and 40.4%, respectively. The use of antimicrobial-based plant by-products of multiple pathogenic bacteria has been reported by numerous researchers (Samavi et al., 2009; Bagewadi et al., 2018; Shricharan and Sivabalan, 2020; Kharat et al., 2020). These results clearly revealed that the plant extracts can be used effectively as bio-pesticides for controlling *Xac* and can replace the chemicals.

Generally, the efforts of evaluating different aqueous extracts from various types of plants to determine their inhibitory action against the citrus canker bacteria either *in vitro* or *in vivo* imply that the adoption and implementation of plant-based toxicants may perhaps become a practical and environmentally sound option for citrus bacterial canker management. The employment of plant extracts for controlling citrus canker organism (*Xac*) yielded some promising results that might pave the way for more serious investigation to avail more options for controlling the disease with low cost and ecofriendly way.

CONCLUSION

The results obtained from this study demonstrated some positive inhibitory effect of roselle (*Hibiscus subdariffa* Linn.) extract against the citrus canker bacterial isolate in both *in vitro* and *in vivo* experiments. The roselle extract needs further investigation *in vivo* at both nursery and orchard levels to be considered as a promising option or an essential IPM component to curtail the spread of citrus bacterial canker disease in Sudan.

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