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Commercial citrus cultivars resistance evaluation and management to canker disease

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Abstract

Citrus production in Pakistan is confronted with a number of biotic and environment stresses due to which yield remained far lower than the potential yield. To combat this problem, a study was conducted in research area of Department of Plant Pathology. Fifteen commercial citrus varieties were screened against canker disease to find out degree of resistance. Jaffa, pine apple, kinnow, mungal singh, tangerine, succari, were found moderately resistant. Five varieties such as chinese lime, musambi, grapefruit, blood red and mayer lime were highly susceptible to canker disease. The susceptible varieties were feutral's early, sweet lime, malta and valentia late showed moderately susceptible response against the canker disease. In vitro and vivo condition, sensitivity to plant extracts and antibiotic of *Xanthomonas axonopodis* pv. *Citri.(Xac)* was studied at different combination. *Withania somnifera* (Aksin), *Achyranthus aspera* (Akk) and Agrimycine-100 (1%) were used at standard dose against *Xac* in vitro. *Withania somnifera* (Aksin), *Achyranthus aspera* (Akk), pesticide (Fon 75%WP, and antibiotic (Agrimycin-100) were used to control citrus canker disease under field conditions. Agrimycine-100 (1%) alone or in combination with *Withania somnifera* (Aksin) performed better against *Xac* produced significantly longer inhibition zones (4.19cm). The combination of Agrimycine-100 at 1% plus *Withania somnifera* at 7.5% and Agrimycine-100 at 1% plus *Achyranthus aspera* significantly reduced the disease compared to control under field condition.

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Introduction

In Pakistan Citrus has an important value as fruit plant. The present day citrus is delectable, juicy and seedless is of great nutritional significance as well (Khan *et al.*, 1992). It is used as best source of Vitamin C, sugars, amino acids and other nutrients (Ahmed and Khan, 1999). Pakistan's economy is based on agriculture and fruit production is always an important part of agriculture. The production of all fruits grew by 3.1 percent, out of which production of citrus fruits grew marginally by 0.1 percent (Anonymous, 2009). Citrus is one of the most important productive and highly profitable fruit crop but unfortunately its present status is threatened by a number of problems including low production induced by pests. Of all the agricultural pests and diseases that threaten citrus crop, citrus canker is one of major diseases which adversely affect plant health and fruit development. Disease incidence increases in the presence of citrus leaf miner (CLM), *Phyllocnistis citrella*. The disease caused by the bacterium *Xanthomonas axonopodis* pv. *citri* (*Xac*)(syn. *X. citri* pv.*citri* (Gabriel *et al.*,1989). The symptoms occurred in the form of necrotic lesions on the leaves, stems and fruits. Severe infections induced defoliation, fruit drop and death of twings (Schoulties *et al.,* 1987). Bacteria also survive for longer periods of time in lesions on woody branches (Goto,1992). Bacteria that ooze on the plant surfaces die within hours from desiccation and exposure to direct sunlight (Graham *et al.*, 2000). Exposed bacteria survive only a few days in soil, and a few months in plant refuse that had been incorporated into soil, (Graham *et al.*, 1989). Because citrus canker reduces the economic gains, so the aim of integrated disease management is to reduce economic losses. Now a day's many diseases of different plants are controlled by various chemicals, but they are adverse to the nature and environment. In the absence of disease resistant varieties, tolerance is useful phenomenon to be used. Keeping in view the great economic importance of citrus canker disease the present study was conducted to identify resistant varieties among the available varieties of citrus against canker and its management through antibiotics and plant extracts.

Materials and Methods

Establishment of disease screening nursery

To evaluate citrus varieties/lines for the relative resistance to canker disease a screening nursery was established in the Plant Pathology Research Area of University of Agriculture, Faisalabad. Varieties encountered for screening were Kinnow(v_1), Pine apple(v_2), Valencia late(v_3), Grape fruit(v_4), Blood red(v_5), Chinese lime(v_6), Mayer lime(v_7), Sweet lime(v_8), Fuetrell's early(v_9), Jaffa (v_{10}), Succari (v_{11}), Tangrin(v_{12}), Mungal Singh (v_{13}), Musambi (v_{14}) and Malta(v_{15}). These varieties were obtained from the fruit plant nursery of Horticulture Department University of Agriculture, Faisalabad. The experiment was laid out under Randomized Complete Block Design (RCBD). Each variety consisted of twelve plants, which were planted in two rows having six plants in each row. All the recommended agronomic practices were followed to maintain citrus nursery in good condition.

Varietal screening against citrus canker disease

Disease incidence was calculated by using the following formula:

$$\text{Disease incidence} = \frac{\text{No. of infected leaves}}{\text{Total no. of leaves}} \times 100$$

Data regarding disease severity was recorded on weekly basis from December 2008 to December 2009. Plants of each variety were randomly selected and disease response was assessed according to (Croxall *et al.*, 1952) disease rating scale (Table 1). Difference in disease incidence among the fifteen varieties was determined by LSD at 5% probability level, Steel *et al.*,1997).

Isolation of bacterium

Leaves exhibiting typical symptoms of citrus canker disease were collected in polyethylene bags and brought to the phytobacteriology laboratory to isolate the bacterium by using the dilution plate technique (Kiralay *et al.*, 1974). First of all, pestles and mortars, petri dishes, medium (Nutrient Agar) and pipettes

were autoclaved at 15 lbs pressure (121°C) for 15-20 minutes. Infected leaf tissues were removed using sterilized 10 mm cork borer. Leaf discs were surface sterilized in 0.1 % mercuric chloride and washed three times in sterilized water. The discs were ground in a sterilized pestles and mortars and the total volume of the mixture adjusted to 10 ml by the addition of sterilized water, followed by the preparation of tenfold dilutions from the mixture. 1 ml of each dilution was poured into a petri dish and Luke warm (45°C) nutrient agar was poured on to it. Each petri dish was shaken. Petri dishes were incubated at 30°C±2°C. Yellow and round colonies appearing after 36 hours incubation were transferred to agar slants to prepare pure cultures. The bacterium was identified using morphological and biochemical characteristics (Breed *et al.,*1989). Stock cultures of the bacterium were maintained on nutrient agar in culture tubes at 4 °C.

Pathogenicity Test

The isolated bacterium was examined for pathogenicity on healthy plants. Two years old ten citrus plants of variety Mayer lemon and Grapefruit were obtained from nursery of Department of Horticulture University of Agriculture, Faisalabad. These plants were transplanted into pots containing field soil disinfected with 5% formalin. The bacterium from stock culture was multiplied on nutrient agar by incubating for 48 hours at 30°C±2°C. An aqueous suspension of the bacterium having a concentration of approximately 108 cells/ml was prepared by plate count method (Kiraley *et al.,*1974).

Just before inoculation, plants were irrigated and covered with polythene bags for two hours to create conditions of high humidity and placed under sunlight to allow the stomata to open to the maximum, (Weindling, 1948; Gunn,1962). The abaxial surface of the leaves was inoculated using a spraying machine at a pressure of 1.1 kg/cm^2 until the tissue showed water soaking. In control the plants were sprayed only with sterilized water. The plants were kept under observation for two weeks in the greenhouse and symptoms, if any were recorded. Reisolation the bacterium from diseased tissue was carried out in the way as described above and morphological characteristics (Breed *et al.,*1989) of the isolates compared with the original culture of bacterium used in inoculations. The bacteria showing similar colony characters that of the original culture were considered to be pathogenic.

Management

In vitro effect of antibiotic and plant extract against Xanthomonas axonopodis pv. citri

Sensitivity to plant extracts and antibiotic of *Xac* was studied. Plant extracts from Withania *somnifera* (Aksin), *Achyranthus aspera* (Akk) were used at standard dose against *Xac.* For the preparation of aqueous extracts, 25 gm fresh leaves of each plant were macerated in 75 ml sterilized water using a sterilized pestle and mortar. The macerated leaf extract was passed through four-layers of sterilized muslin cloth and filtered through Whatman filter paper No. 41. The extract obtained was considered standard (S) arbitrarily and was stored at -20 °C until use. A bacterial suspension containing approximately 10^8 cfu/ml of *Xac* was prepared from a 48 hours old culture as described previously. The suspension was mixed with the Luke warm nutrient agar at a concentration of 1ml/25 ml of media poured in sterilized Petri dishes. Petri dishes were gently shaken to mix the bacteria uniformly in the nutrient agar and then allowed to solidify. Wells (1 cm dia.) were made into the agar using a sterilized 1 cm diameter cork borer and plant extracts and antibiotics were pipetted into these wells. All Petri dishes were placed in refrigerator at 4°C for 24 hours before transfer to an incubator at 28 ± 2°C for 48 hours. The experiment was conducted in Completely Randomized Design (CRD) with three replications per treatment. Control cultures had sterilized water in the wells. The data recorded on inhibition zones were subjected to analysis of variance and treatment means were compared by LSD (Steel *et al.*, 1997).

T_1 = Agrimycine-100 (1%) T_2 = *Withania somnifera*(S*)

T_3 = *Achyranthus aspera* (S*)

T_4 = Agrimycine- 100 (.1%) +*Withania somnifera* (S*)

T_5 = Agrimycine- 100 (.1%) +*Achyranthus aspera* (S*)

T_6 = Control

S* = Standard dose.

Management of citrus canker disease under field conditions

Plant extracts from *Withania somnifera* (Aksin), *Achyranthus aspera*

(Akk) and antibiotic (Agrimycin-100) were used to control citrus canker disease under field conditions. Sterilized water was used as control. For this purpose experiments was conducted under the field conditions to control citrus canker disease. Data regarding disease incidence were recorded before and after application of treatments. There were three replications of each treatment including control and subjected to analysis of variance. The treatments were applied according to following plan;

Experiment #

T_1 = Agrimycine-100(1%)

T_2 = *Withania somnifera* (15%)

T_3 = Agrimycine-100(1%) + *Withania somnifera* (7.5%)

T_4 = *Achyranthus aspera* (15%)

T_5 = Agrimycine-100(1%) + *Achyranthus aspera* (7.5%)

T_6 = Control.

Results

Reaction of different citrus cultivars/varieties against canker disease

Fifteen varieties/lines of citrus were evaluated for relative resistance to canker disease in natural environment. All the varieties showed different levels of disease development. *C. limonia* cv. china lemon, *C. sinensis* cv. Succari, *C. paradise* cv. grapefruit, *C. sinensis* cv. blood red and *C. limonia* cv. mayer lemone were highly susceptible with disease ratings of 9. while *C. reticulata* cv. feutral's early, *C. reticulata* cv. malta and *C. limettioides* cv. sweet lemon were susceptible with disease ratings of 7.

Table 1. Disease rating scale used to determine the level of resistance or susceptibility to citrus canker.

Grade	*Disease Severity (%)*	Response
0	00-00	Highly Resistant
1	01-05	Resistant
3	06-10	Moderately Resistant
5	11-15	Moderately Susceptible
7	16-25	Susceptible
9	26 and above	Highly Susceptible

C. sinensis cv. valentia late showed moderately susceptible response against the disease with disease rating of 5 while *C. sinensis* ev. jaffa, *C. sinensis* cv. pine apple, *C. reticulata* cv. tangerine, *C. reticulata* cv. kinnow, *C. sinensis* cv. succari, *C. reticulate* cv. mungal singh were moderately resistant with disease ratings of 3. (Table 2).

Citrus Canker Disease Management

In vitro effect of antibiotic and plant extracts against Xanthomonas axonopodis pv. Citri

For in vitro management of *Xac*, Agrimycine-100 at 1% and *Withania somnifera, Achyranthus aspera* at standard dose were tested. The combination of Agrimycine-100 at 1% and plant extract at standard dose were also checked against *Xac.* Agrymicine-100 at 1% reduced the multiplication of *Xac* significantly compared to control and produced significantly longer inhibition zones (4.19cm) compared to other tested treatments. Antibiotic alone or in combination with *Withania somnifera* (Aksin) performed better against *Xac*, *Achyranthus aspera* (Akk) was the least active plant extract tested against *Xa* (Table 4).

Management of citrus canker disease under field conditions through antibiotic and plant extracts

The treatments Agrimycine-100 at 1%, *Withania somnifera* at 15%, *Achyranthus aspera* at 15%,

Agrimycine-100 at 1% plus *Withania somnifera* at 7.5% and Agrimycine-100 at 1% plus *Achyranthus aspera* at 7.5% concentration applied to determine their efficacy against citrus canker disease under field conditions. The combination of Agrimycine-100 at 1% plus *Withania somnifera* at 7.5% and Agrimycine-100 at 1% plus *Achyranthus aspera* significantly reduced the disease compared to control (Table 6).

Table 2. Level of resistance/susceptibility to canker disease exhibited by various citrus varieties.

Sr.#	Varieties/cultivars	Disease incidence (Mean)	Ratings	Response
1	Jaffa	8.01 k*	3	MR
2	Pine apple	8.29 k	3	MR
3	Kinnow	8.60 jk	3	MR
4	Mungal singh	8.98 ij	3	MR
5	Tangerine	9.52 hi	3	MR
6	Succari	9.78 h	3	MR
7	Valentia late	10.90 g	5	MS
8	Feutral'early	18.06 f	7	S
9	Sweet lime	20.86 e	7	S
10	Malta	20.97 e	7	S
11	Chinese lime	25.73 d	9	HS
12	Musambi	27.83 c	9	HS
13	Grapefruit	30.15 b	9	HS
14	Blood red	30.37 b	9	HS
15	Mayer lime	31.50 a	9	HS

*Means sharing similar letters do not differ significantly (P>0.05).

MR = Moderately resistant

MS = Moderately susceptible

S = Susceptible

HS = Highly susceptible.

Discussion

Citrus canker disease, caused by *X. axonopodis* pv. *citri* has re-emerged as potential threat to citrus plantation throughout the world including Pakistan, (Civerolo, 1984). The citrus cultivars previously known to be resistant to this pathogen have now become susceptible. Once this disease becomes endemic in an area, it is very difficult to manage with commercially acceptable methods under favorable conditions for disease development (Das, 2003). Genetic resistance probably is the only durable and long lasting solution to citrus canker disease. The short-term solution should be screening of available germplasm for relative susceptibility, as in this study and to identify low rating variations for breeding manipulation.

Out of 15 cultivars *C. sinensis* ev. jaffa, *C. sinensis* cv. pine apple, *C. reticulata* cv. tangerine, *C. reticulata* cv. kinnow, *C. sinensis* cv. succari, *C. reticulata* cv. mungal singh exhibited resistat response, whereas *C. limonia* cv. china lemon, *C. sinensis* cv. succari, *C. paradise* cv. grapefruit, *C. sinensis* cv. blood red and *C. limonia* cv. mayer lemone showed high susceptibility to citrus canker disease. (Table 2).

Evaluation of different varieties of citrus to find resistant source against canker has been reported by many research workers and the results of present study were agreed (Wang and Chung, 1991) who observed that *Xac* occurred widely on grapefruit, sweet orange, lemon and other citrus species. (Ayub *et al.,* 1996) concluded that isolates of *Xac* when inoculated into various citrus hosts, *C. aurantifolia, C.* aurantrium, *C. paradisi, C.* limon, *Ponicirus trifoliata* and *C. sinesis,* these hosts showed susceptibility in decreasing order. *C. reticulata* was found to be resistant. (Civerolo, 1984) reported that

among commercial citrus varieties and rootstocks, Asiatic citrus canker (ACC) was most severe on *C. paradise, C. aurantifnlia, C. limettioides, Poncirus trifoliata* and their hybrids because of high susceptibility.

Table 3. ANOVA for in vitro evaluation of Agrimycine-100 and plant extracts against *Xanthomonas axonopodis* pv. *Citri.*

SOV	DF	SS	MSS	F-value
Treatments (T)	5	119.249	23.850	959.75**
Dose (D)	2	0.397	0.198	7.99**
Interaction (T x D)	10	1.140	0.114	4.59**
Error	36	0.895	0.025	
Total	53	121.680		

** = Highly significant (P<0.01).

(Leite and Mohan, 1990) reported that there was a wide range of variability for resistance to citrus canker disease in the citrus germplasm. The commercial citrus canker - resistant cultivars of sweet orange, mandarins and tahiti lime are usually recommended for planting. According to (Pavan *et al.* 2007), mandarins and tangerines were recognized as tolerant to Asiatic citrus canker disease while Sweet orange exhibited the susceptible response among commercial varieties. The results of screening were also matched with that of (Atiq *et al*., 2007) screened fifteen citrus cultivars for the source of resistance against citrus canker disease incited by (*Xanthomonas campestris* pv. *citri*) and concluded that *Citrus sinensis* ev. jaffa exhibited resistance response while *Citrus paradise*, *Citrus sinensis* cv. blood red, *Citrus limonia* cv. mayer lemon showed highly susceptible expression. *Citrus sinensis* cv. valentia late, *Citrus reticulata* cv. feutral's early showed moderately resistant while *Citrus reticulata* cv. malta, *Citrus limettioides*, *Citrus limonia* cv. china lemon, *Citrus sinensis* cv. musambi were found moderately susceptible toward canker disease. No citrus cultivar was found immune.

Table 4. In vitro effect of Agrimycine-100 and plant extracts against*Xanthomonas axonopodis* pv. *Citri.*

Sr. #	Treatments	Mean values of inhibition zones (cm)
T_1	Agrimycine-100 (1%)	4.19 a
T_2	*Withania somnifera* (S*)	1.87 d
T3	*Achyranthus aspera* (S*)	1.31 e
T4	Agrimycine-100(.1%) +*Withania somnifera* (S*)	3.77 b
T5	Agrimycine-100(.1%) +*Achyranthus aspera* (S*)	3.37 c
T_6	Control	0.00 f
	LSD	0.151

Means sharing similar letter are statistically non-significant (P>0.05).

Plant extracts and the antibiotic, Agrimycin-100 varied greatly in effect on the growth of *Xac*. Agrimycin-100 at 1% and combination of Agrimycine-100 at .1% plus plant extract (*Withania somnifera, Achyranthus* aspera) at standard dose in vitro proved to be effective, whereas *Achyranthus aspera* extract tested alone proved less effective in the inhibition of the bacterial culture at standard dose(Table 4). Antibiotic singly or in combination with plant extracts inhibited the *Xac*. Agrimycin-100 and plant extracts were also applied to control the citrus canker disease under field condition. Agrimycin-100 at 1% in combination with plant extracts (*Withania somnifera, Achyranthus aspera)* at 7.5% concentration reduced the disease compared to control. Among plant extract *Withania somnifera* at 15%, was more effective against *Xac* as compared to *Achyranthus aspera* at 15%. (Table 6).

Table 5. ANOVA for evaluation of Agrimycine-100 and plant extracts to control the citrus canker disease under field conditions.

SOV	DF	SS	MSS	F-value
Replication	2	1.083	0.541	0.15
Treatments (T)	5	2353.841	470.768	126.24**
Days (D)	2	669.086	334.543	89.71**
Interaction (T x D)	10	77.734	7.773	2.08^{Ns}
Error	34	126.791	3.729	
Total	53	3228.534		

Withania somnifera and *Achyranthus aspera and* antibiotic were capable of reducing the growth of *Xac* in vitro and in vivo. These results were agreed to Mosses and Chandramohan (1993), who tested 16 crude plant extracts in vitro against *Xac and* reported that extracts of Neem cake, Neem leaf and *A. salivum* (garlic) extracts were effective against *Xac*. Both Vaheeuddin *et al.* (1957) and Reddy and Papa (1960) demonstrated that Neem cake in suspension effectively checked citrus canker disease.

Table 6. Effect of Agrimycine-100 and plant extracts to control the citrus canker disease under field conditions.

Sr. #	Treatments	Mean values of citrus canker disease incidence
T1	Agrimycine-100(1%)	27.37 b
T_2	*Withania somnifera* (15%)	21.01 d
T3	Agrimycine-100(1%) + *Withania somnifera* (7.5%)	14.23 f
T_4	*Achyranthus aspera* (15%)	23.67 c
T_5	Agrimycine-100(1%) + *Achyranthus aspera* (7.5%)	16.74 e
T_6	Control	34.06 a
	LSD	1.850

Means sharing similar letter are statistically non-significant (P>0.05).

(Sahi *et al.,* 2007) found that Agrimycin -100 was most effective as compared to other toxicants which were tested in vitro and in vivo against *Xac.* (Pereira *et al.,* 1981) applied streptomycin plus dehydro streptomycin at 200 g/ha and achieved good results in controlling citrus canker disease.

Conclusion

Control of citrus canker in the areas where disease is present, the most effective disease management strategy is the use of disease resistant varieties, including timely application of protective, bio-chemicals and plant extracts are recommended because frequent use of chemicals is neither economical nor beneficial for the environment.

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