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# EFFICACY OF CITRUS ROOTSTOCKS AGAINST CITRUS CANKER (*XANTHOMONAS AXONOPODIS* PV. *CITRI*) INFESTATION

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**A B S T R A C T**

The study was conducted to find out degree of resistance in different citrus rootstocks against citrus canker (*Xanthomonas axonopodis* pv. *citri*) infestation. Nine different citrus rootstocks were evaluated against citrus canker disease resistance. The role and profile of proteins, proteases, sugars, nitrogen and total phenolic contents were described in the incidence of disease. Disease rating scale having 0-4 range was used to distinguish among resistance and susceptibility of rootstock. Out of nine rootstocks; three rootstocks (Fraser hybrid, Cox mandarin hybrid and C-35) were resistant, three were found moderately susceptible (*Poncirus trifoliata*, *Troyer citrange* and *Carrizo citrange*) and three (*Cleopatra mandarin*, Rough lemon and Benton) were susceptible. Biochemical analysis revealed that there was an increasing trend in the citrus canker disease incidence with total sugars contents while in case of total phenolic contents, protein and protease activity, decreasing trend was observed in disease incidence in all the rootstocks. It is suggested that the resistant rootstocks could be used in breeding to produce the resistant citrus cultivars against citrus canker disease.

**Keywords**: citrus canker, rootstocks, protein, phenolics (TPC), sugars, protease

## INTRODUCTION

Citrus is recognized as an agricultural commercial fruit and occupies a distinguished position in the fruit production. It stands as the second most important fruit worldwide after grapes in terms of area and production (Anonymous 2005). Pakistan stands among eminent citrus producers of the world (Anonymous 2005). In 2014, 2.17 million tones of citrus was produced on area of 1.94 million acres in Pakistan (Anonymous, 2014). Although citrus is kept in great esteem yet present status is threatened by a number of factors which hamper the quantity and quality of fruit (Burhan *et al.,* 2007). The pests and diseases that threaten the citrus crops, canker is one of the most devastating diseases affecting citrus worldwide (Das, 2003). In Pakistan, this disease is prevalent throughout the country. This disease is caused by the bacterium *Xanthomonas axonopodis* pv. *citri.* The bacteria form lesions on leaves and fruits (Hussain *et al.*, 1988). Intensity of infection varies with the species and cultivars (Falico-De-Alcaraz, 1986). Burhan *et al.*,(2007) analyzed the disease resistance variation in 15 cultivars of oranges and noted that different cultivars have different disease resistance. Citrus trees consist of two parts, the rootstock and the scion. Rootstocks greatly influence characters of variety as it ensures tolerance to abiotic and biotic stress conditions. So, rootstock selection is one of the most important decisions a grower makes when establishing commercial citrus orchards. The rootstock has several major responsibilities like provision of level of resistance against diseases. Selection of resistant varieties is the most efficient method to combat diseases. Therefore, this experiment was conducted to screen out resistant rootstocks against citrus canker. In Pakistan previously no work had been done on citrus rootstocks. The

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outcomes of this study will be helpful in breeding for selection of resistant rootstock against citrus canker to boost up citrus industry.

This paper reports the response of selected rootstocks against citrus canker disease (*Xanthomonas axonopodis* pv. *citri)* and the role of proteins, proteases, sugar contents, nitrogen and total phenolic contents in the disease incidence.

**MATERIAL AND METHODS**

Nine different citrus rootstocks viz. Cleopatra mandarin (*Citrus rashni*), Poncirus trifoliate (*Poncirus trifoliata*), Fraser hybrid, Cox mandarin hybrid, Troyer citrange (*Citrus sinensis × Poncirus trifoliata*), Carrizo Citrange (*Citrus sinensis × Poncirus trifoliate*), Benton (*Citrus sinensis × Poncirus trifoliata*), C-35 (Rubby orange × *Poncirus trifoliata*) and Rough lemon (*Citrus jambhiri* Lush) were taken from the Citrus Sanitation Nursery, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan.

The pots (09 inches size) containing susceptible rootstocks of rough lemon were served as control. Five plants of each rootstock were selected randomly of 2 years age and tagged for recording disease index. All the plants were 1.5-2 ft in height. These plants were kept in glass house under controlled conditions by using completely randomized design. Leaves revealing distinctive symptoms of citrus canker were collected in the polythene bags and isolated bacterium by using the dilution plate technique (Kiralay *et al.,* 1974) in Citrus Sanitation Lab., Institute of Horticultural Sciences, University of Agriculture Faisalabad, Pakistan. A special medium known as Nutrient Agar (Khan & Chohan 2000) was used for isolation of bacterium. The isolated organism was purified and multiplied which was identified as *Xanthomonas axonopodis* pv. *citri*. on the basis of morphological and biochemical characteristics (Breed *et al*., 1957) The stock culture of bacterium was maintained on nutrient agar in culture tubes at 4°C in refrigerator. All the rootstocks were inoculated with *Xanthomonas axonopodis* pv. *citri*. Just before the inoculation, plants were irrigated and covered with polythene bags to create conditions of high humidity and placed under sunlight for maximum opening of stomata. The abaxial surface of the leaves was inoculated by using a spray machine at a pressure of 1.1 kg/cm$^2$ until the leaves soaked. Disease severity of leaves was recorded after 21 days of inoculation. Percentage of diseased leave was calculated for each rootstock by using following formula:

$$\text{Diseased leaves\%} = \frac{\text{No. of infected leaves}}{\text{Total No. of leaves}} \times 100$$

The level of resistance or susceptibility in each rootstock was determined according to Croxal (1952) by using the scale described in Table 1.

Table 1. Scale to test the level of sensivity against citrus canker.

| Grade | Disease severity% | Response |
|---|---|---|
| 0 | 00-00 | Immune |
| 1 | 01-05 | Resistant |
| 3 | 06-10 | Moderately Resistant |
| 5 | 11-15 | Moderately Susceptible |
| 7 | 16-25 | Susceptible |
| 9 | 26 and above | Highly Susceptible |

( Horsfall and Heuberger, 1942)

**BIOCHEMICAL ANALYSIS**

**Protein determination:** Protein of the samples was determined by Bradford method (Bradford, 1976). Diseased leaves were ground in pestle and mortar and powdered sample was homogenized in potassium phosphate buffer (pH 7.7). The homogenate paste was centrifuged at 10,000 xg for 5 min. Each sample, 100 µL, was taken in a test tube and 1.0 mL of Bradford reagent (BioRad) was added. The sample solutions were incubated at 37°C for 10-20 minutes (ISUZU, Japan) along with the blank and absorbance was noted at 595 nm. Protein concentration was determined by using bovine serum albumin (BSA) as standard.

**Quantitative estimation of protease activity:** Protease activity was determined by casein digestion method described by Drapeau (1974). Tubes were equilibrated with 2.0 mL of 1% casein (sigma) at 37°C for 5 min. 100 µL of protease extract was added to all the tubes and mixed well. A reagent blank was also included. After 10 min of adding samples, reaction was stopped by adding 2.0 ml (10%) TCA solution and mixed well. Tubes were allowed to stand for 10 min and the reaction solution

was filtered to remove the precipitate formed during the reaction. The absorbance of the filtrate was measured at 280 nm on spectrophotometer (Hitech, Japan).

**Total Soluble Sugars (TSS):** Total soluble sugars were determined by rapid and convenient Anthrone reagent method reported by Thimmaiah (2004). Sample of 50 µL was hydrolyzed with 2.5 N HCl for 3 h in boiling water bath. Cooled and neutralized it with solid $Na_2CO_3$ and made up the volume to 10 mL. In 1 mL of that sample added 5% phenol reagent and 5 mL of 95% sulphuric acid and heated for 10 min in water bath at 30°C. Cooled and read the orange-yellow colour at 490 nm, calculated the concentration of total soluble sugars by using glucose as standard.

**Total Phenolic Contents (TPC):** TPC were determined using Folin-Ciocalteu reagent method as described by Oviasogie *et al*. (2009). Leaves were stored at -80°C and homogenized in cold pestle and mortar and incubated the sample at room temperature for 48 hours in the dark. Then centrifuged the sample at 10,000 X g for 5 minutes and supernatant was separated. Added 100 µL supernatant and 200 µL of Folin-Ciocalteu reagent and vortex thoroughly. The Folin Ciocalteu reagent was added before the alkali to avoid the air oxidation of phenols. Then added 800 µL 700 mM $Na_2CO_3$ in each tube and incubated the assay at room temperature for 2 hour and transferred the 200 µL sample from the assay tube to a clear 96 well microplate and read the absorbance of each well at 765 nm. The results are expressed as GAE (gallic acid equivalents g/100g) of dry matter.

**RESULTS AND DISCUSSION**

Inoculation with *Xanthomonas axonopodis* pv. *citri*. produced well developed canker lesions within 21 days. The results presented in Table 2 depicts that there were considerable differences among different rootstocks for the level of resistance and susceptibility against the disease. The highest value was for Rough lemon (23.5%), followed by Benton (19.7%), Cleopatra mandarin (18.5%), Carrizo citrange (13.5%), Poncirus trifoliata (12.6%), Troyer citrange (11.5%), C-35 (3.7%), Fraser hybrid (3.2%) and Cox mandarin hybrid (2.8%).

Cox madarin hybrid, Fraser hybrid and C -35 showed resistant against citrus canker disease while Poncirus trifoliata, Troyer citrange and Carrizo citrange were moderately susceptible to citrus canker. Rough lemon, Cleoptra mandarin and Benton were susceptible to the disease. No rootstock was found immune to the citrus canker disease.

In this context the results are in consistence with Kale *et al.* (1996) who reported that the incidence of citrus canker and the results showed that trifoliate orange (*Poncirus trifoliata)* was less susceptible to citrus canker (*Xanthomonas axonopodis* pv. *citri*) than acid lime and rough lemon as in our results the disease incidence was more in Rough lemon (23.5%) than Poncirus trifoliata (12.60%).

Table 2. Sensitivity of nine different citrus rootstocks against canker disease.

| Variety | Disease index % | Response |
|---|---|---|
| Cleopatra Mandarin | 18.5 | S |
| Poncirus Trifoliata | 12.60 | MS |
| Fraser Hybrid | 3.20 | R |
| Cox Mandarin Hybrid | 2.80 | R |
| Troyer Citrange | 11.50 | MS |
| Carrizo Citrange | 13.50 | MS |
| Benton | 19.70 | S |
| C-35 | 3.7 | R |
| Rough Lemon | 23.5 | S |

S-Susceptible; MS-Moderately susceptible; R-Resistant

**Protease (IU/mg of protein) specific activity and disease index:** The data regarding protease activity of different citrus rootstocks was collected and analyzed according to the complete randomized design. The means were arranged in a descending order and Least Significant Difference (LSD) test was applied to separate the means at 5% probability. The data pertaining to means for protease specific activity (Table 3) indicated that the protease specific activity ranged of 0.12-0.30 IU/mg of protein. Maximum value was recorded for the Cox mandarin hybrid (0.30 IU/mg of protein) followed by Fraser hybrid and C-35 having the values 0.28 IU/mg of protein and 0.25 IU/mg of protein, respectively. The minimum value (0.12 IU/mg of protein) was observed in

Rough lemon. There was a negative relation of the citrus canker disease occurrence with protease activity as shown in Figure 1(a).

These results are in agreement with Barrett 1994 that reported proteases are end peptidases that preferentially hydrolyze internal peptide bonds in polypeptide chains. Proteases are protein hydrolyzing enzymes and are implicated to play an important role in the disease resistance. The protease specific activity was more in the leaves of resistant rootstocks than moderately susceptible or susceptible rootstocks. Disease index was maximum in Rough lemon (23.5%) and protease activity was found minimum in Rough lemon (0.12 IU/mg of protein). Cox mandarin hybrid had the minimum disease index (2.8%) and the protease activity was found maximum (0.3 IU/mg of protein). There was more protease activity in those rootstocks which showed gradually less disease.

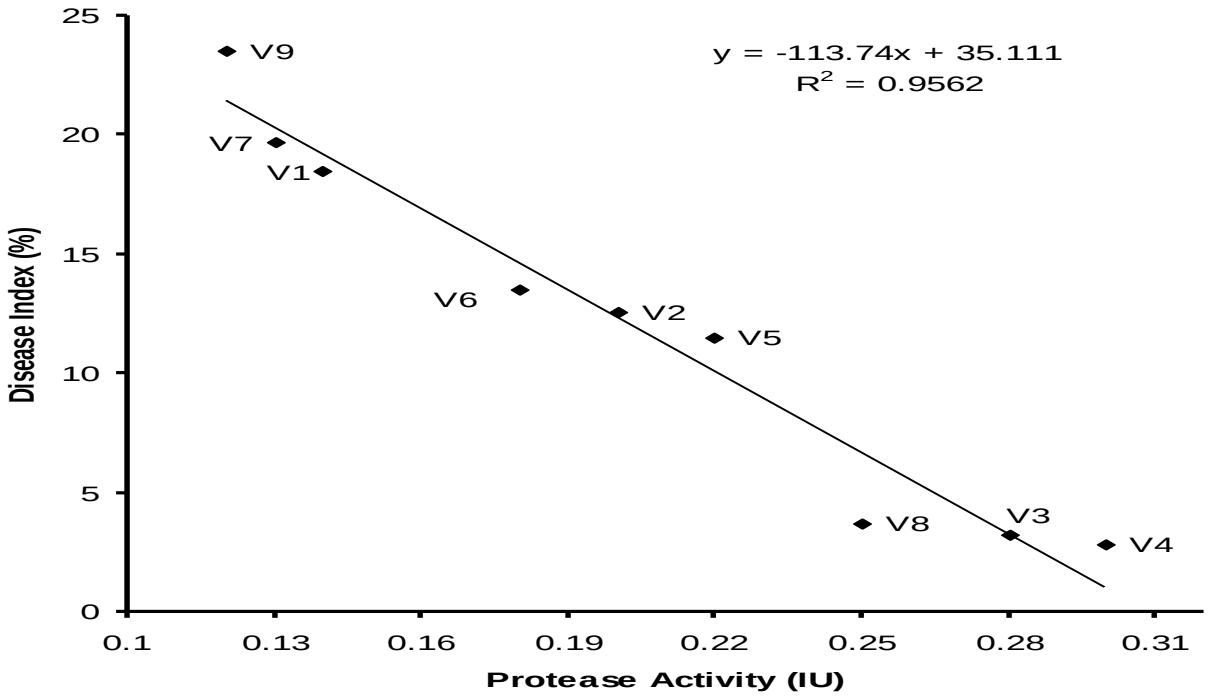


Figure 1. (a) Relationship between canker disease index and protease specific activity in nine citrus rootstocks.

**Protein estimation (µg/mL):** The data regarding proteins present in different citrus rootstocks was collected and analyzed according to the complete randomized design. The mean values of protein present in different rootstocks indicated that the protein ranged 790.49-159.11 µg/mL (Table 3). Minimum value was recorded for the Rough lemon and maximum for the Cox mandarin hybrid. For Fraser hybrid, C-35, Troyer citrange, Carizo citrange and Benton, the values of proteins were 715.45µg/mL, 546.1 µg/mL, 438.15 µg/mL, 349.47 µg/mL and 216.57 µg/mL, respectively.

Plant pathogens like viruses, bacteria, nematode and fungi elicit synthesize of host proteins, pathogenesis related proteins which help in restricting the multiplication and spread of pathogens in healthy tissues. The results revealed that negative relationship existed between protein contents and disease index in citrus rootstocks (Figure 1b). The protein contents were more in the leaves of resistant rootstocks than moderately susceptible or susceptible rootstocks. Actually when the plant goes under stress, it produces the protein having defensive role against the pathogens. This is in agreement with previous findings of other research workers (Niedz *et al.*1994).

Disease index was maximum in Rough lemon (23.5%) and protein contents were found minimum in Rough lemon (159.11 µg/mL). Cox mandarin hybrid had the minimum disease index (2.8%) but the protein contents were found maximum (790.49 µg/mL). It is concluded that resistant varieties showed more protein contents than the susceptible varieties. There was more protein content in rootstocks which showed gradually less disease symptoms.

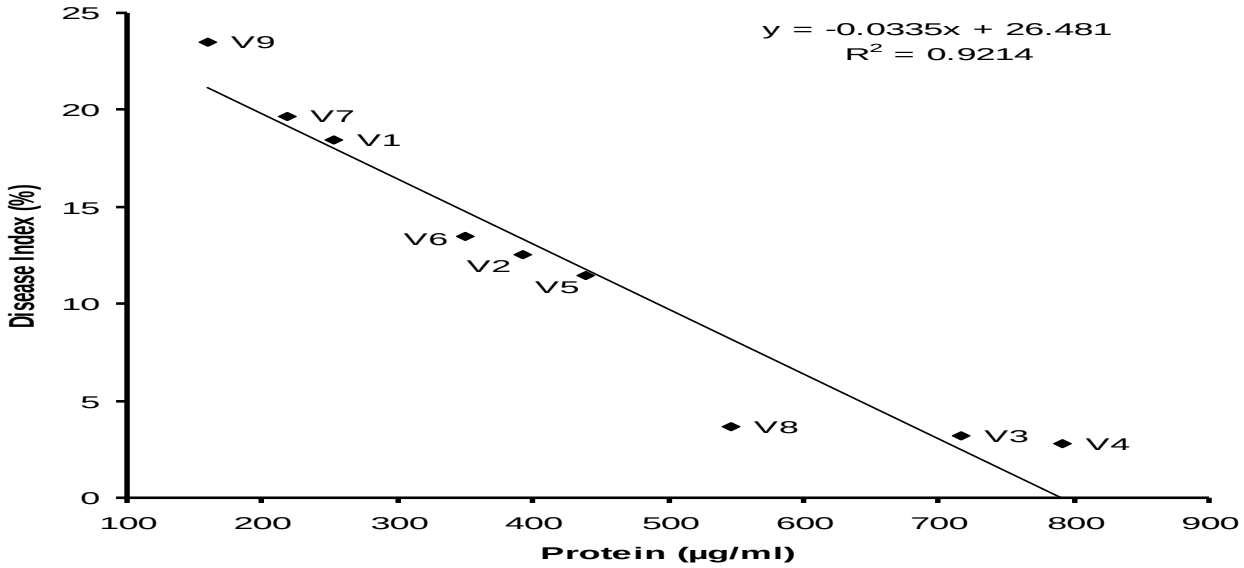


Figure 1: (b) Relationship between canker disease index and protein contents in nine citrus rootstocks.

**Total soluble sugars (TSS) (mg/g) and disease index:**

The data regarding total soluble sugars present in different citrus rootstocks was collected and analyzed according to the complete randomized design The means for total soluble sugars in Table 3 expounded that the Rough lemon has the maximum value (20.9 mg/g) and has the significant effect, followed by the Benton (19.49 mg/g), Cleopatra mandarin (18.34 mg/g) and Carizo citrange (16.22 mg/g); however, the lowest (8.82 mg/g) value was observed for Cox mandarin hybrid.

The results revealed that a relationship existed between total sugars in the leaves and disease index. There was a positive correlation in the citrus canker disease incidence and total sugars as shown in Figure 1c.

Our results confirmed the findings of Pullaiah *et al.*1993 who found that susceptibility was positively correlated to total sugars as the total sugars were higher in the leaves of susceptible rootstocks than moderately susceptible and resistant rootstocks. Disease index, total sugars were also found maximum in Rough lemon (23.5% and 20.9 mg/g respectively).

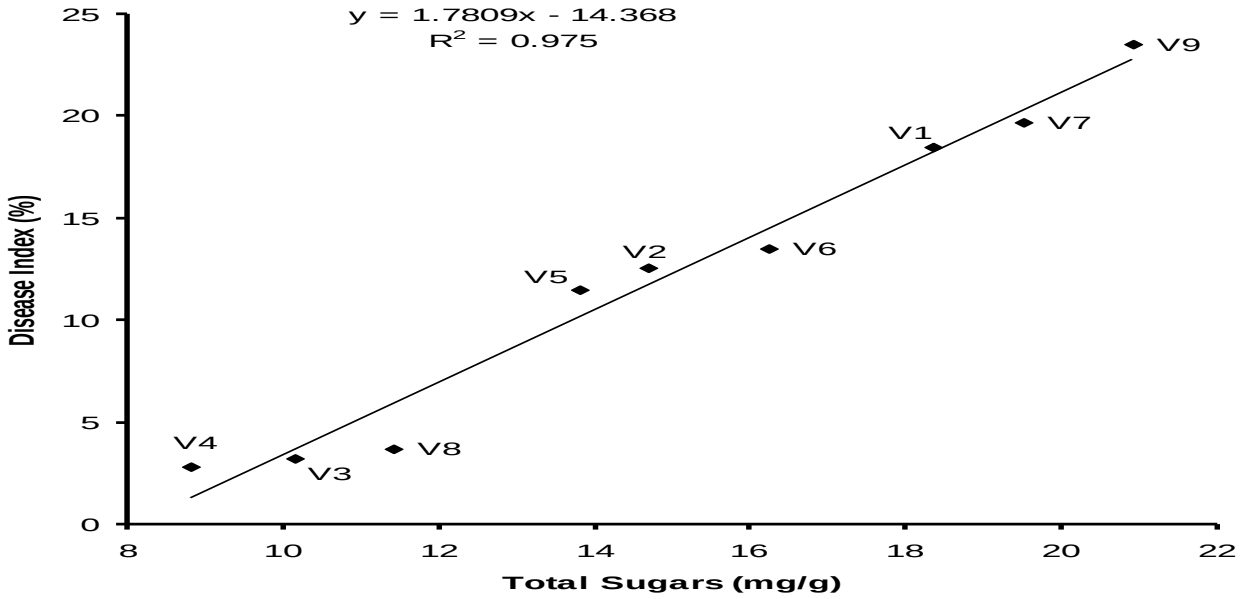


Figure 1. (c) Relationship between canker disease index and total soluble sugars in nine citrus rootstocks.

**Total phenolic contents (TPC) (mg/g):** The data regarding total phenolic contents present in different citrus rootstocks was collected and analyzed according to the complete randomized design. Mean values regarding total phenolic contents showed maximum values for the Cox mandarin hybrid (22.99 mg/g) and Fraser hybrid (22.93 mg/g) and the lowest phenolic contents was recorded in the Rough lemon (4.5 mg/g) (Table 3).

There was a negative correlation of the citrus canker disease incidence with total phenolic contents (Figure 1d). The total phenolic contents were more in the leaves of resistant rootstocks than moderately susceptible and susceptible rootstocks. Disease index was maximum in Rough lemon (23.5%) and total phenolic contents were found minimum in Rough lemon (4.5 mg/g). Cox mandarin hybrid had the minimum disease index (2.8%) but the total phenolic contents were found maximum (22.93 mg/g). It was concluded that resistant varieties exhibited higher concentrations of phenolics than susceptible. This is in agreement with previous findings of other research workers (Abid *et al.*2008).

It was found that resistant rootstocks showed higher levels of total phenolic content than the susceptible rootstocks. There were more total phenolic contents in rootstocks which showed the gradually less disease incidence. Previous reports confirmed that increase in the production of phenolic compounds restrict the pathogen invasion (Nicholson and Hammerschmidt, 1992). It is also confirmed that some of the phenols in healthy part of the plant may act as antimicrobial activity while those that are induced in the response of pathogen attack have proven themselves as a defender against disease in chickpea (Cherif *et al*., 2007).

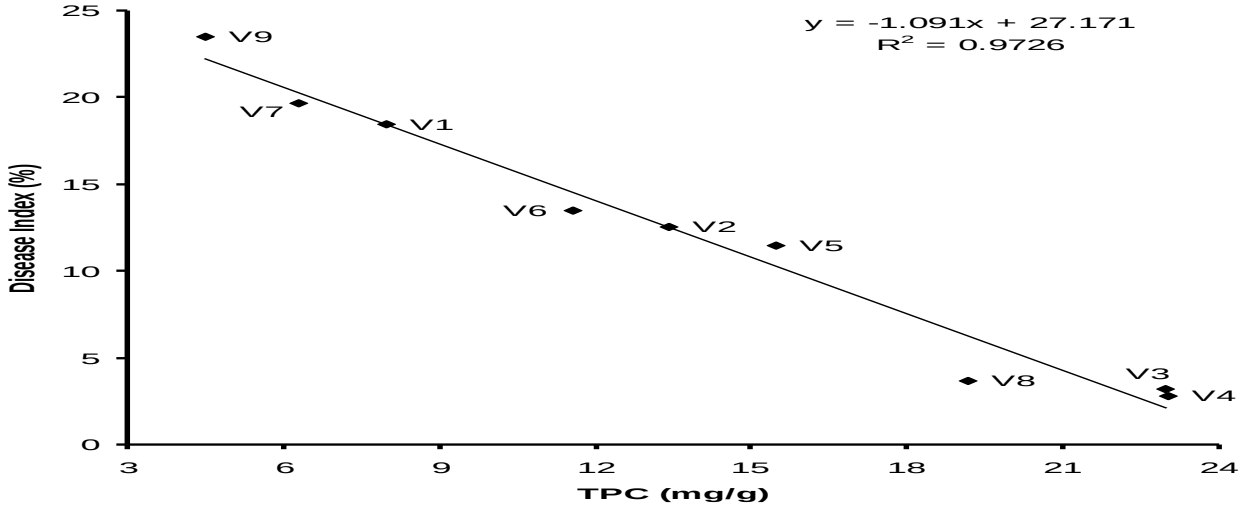


Figure 1. (d) Relationship between canker disease index and total phenolic contents in nine citrus rootstocks.

Table 3. Level of Total phenolic contents, Protein, Total sugars, Nitrogen percentage and protease specific activity in selected nine citrus rootstocks.

| Treatments | TPC (mg/g) | Proteins (µg/mL) | Total sugars (mg/g) | Protease (IU/mg of protein) |
|---|---|---|---|---|
| Cleopatra mandarin | 7.98±0.481 | 252.49±9.104 | 18.34±0.364 | 0.14±0.001 |
| Poncirus trifoliate | 13.42±0.486 | 390.67±17.26 | 14.7±0.484 | 0.20±0.008 |
| Fraser hybrid | 22.93±0.906 | 715.45±12.39 | 10.14±0.350 | 0.28±0.007 |
| Cox mandarin hybrid | 22.99±0.708 | 790.49±17.38 | 8.82±0.243 I | 0.30±0.007 |
| Troyer citrange | 15.47±0.404 | 438.15±14.61 | 13.79±0.221 | 0.22±0.003 |
| Carizo citrange | 11.53±0.484 | 349.47±9.132 | 16.22±0.223 | 0.18±0.004 |
| Benton | 6.25±0.712 | 216.57±3.959 | 19.49±0.403 | 0.130.0.001 |
| C-35 | 19.16±0.410 | 546.1±25.415 | 11.41±0.161 | 0.25±0.003 |
| Rough lemon | 4.50±0.512 | 159.11±5.442 I | 20.9±0.125 | 0.12±0.001 |
| LSD | 0.7570 | 18.267 | 0.3944 | 6.33 |

Mean ± SD. The data presented in above table is mean of three independent parameters.

## CONCLUSION

It is concluded from the contemporary studies that three rootstocks (Fraser hybrid, Cox mandarin hybrid and C-35) were found resistant against citrus canker. This study will help the breeders to use the resistant rootstocks in breeding program to produce the resistant citrus cultivars against citrus canker disease and the biochemical compound as biomarker.

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## REFERENCES

Abid, M., M.A. Khan and A. Wahid. 2008. Screening of citrus cultivars and determination of phenolics in relation to resistance against citrus canker. Pak. J. Pathol. 20(2): 180-184.

Anonymous. 2005. FAO Production year book, Food and Agriculture Organization of the United Nations, Rome Italy

Anonymous. 2014. Pakistan statistical yearbook. Federal Bureau of Statistics, Ministry of Economic Affairs and Statistics, Government of Pakistan, Islamabad.

Barrett, A.J. 1994. Classification of peptidases. Method Enzymol. 244: 115pp.

Bradford, M.M. 1976. A rapid and sensitive method for the quantities of protein utilizing the principle of protein-dye binding. Ann. Biochem. 72: 248-254.

Breed, R.S., E.G.D. Murry and N.R. Smith. 1957. Bergeys Mannual of Determinative, Bacteriology, Williams and Eilinson Company, Baltimore, 7th ed.165 pp.

Chérif, M., Arfaoui and A. Rhaiem. 2007. Phenolic compounds and their role in Bio-control and resistance of chickpea to fungal pathogenic attacks. Tunis J. Plant Prot. 2(1): 7-22.

Cuenca, M. R., M, A. P. Capella, and M. A. F Gine. 2016. Influence of Rootstock on Citrus Tree Growth: Effects on Photosynthesis and Carbohydrate Distribution, Plant Size, Yield, Fruit Quality, and Dwarfing Genotypes

Das, A.K. 2003. Citrus canker – A review. J. Appl. Hort. 5(1): 52-60.

Drapeau, G. 1974. Protease from Staphylococcus aureus. *In:* Lorand L. (ed.). Methods in Enzymology. 45B. Acad. Press., NY. 469 pp.

Falico-de-Alcaraz, L. 1986. Multiplication of *Xanthomonas compestris. pv. citri* in leaf tissue of different citrus species. Phytopathologia. 21: 52-60.

Horsfall, J.G. and J.W. Heuberger. 1942. Measuring magnitude of defoliation disease of tomatoes. Phytopathol. 32: 226-232.

Hussain, M., A.S. Akhtar and M.Z. Iqbal. 1988. Survey of citrus disease in selected orchards of the Punjab. AARI, Faisalabad. 11 pp.

Kale, K.B., J.G. Raut and Y.H. Dudhe. 1996. Influence of rootstocks on the incidence of bacterial canker of acid lime. P.K.V. Res. J. 20(1): 105-106.

Khan, M.A. and R.A. Chohan. 2000. Laboratory manual for bacterial plant pathology.

M.Sc. Thesis. Deptt. of Plant Pathol., Univ. of Agric., Faisalabad. Pakistan.

Kiralay, Z., Z. Klement, F. Elaymasy and J. Vaaras. 1974. Methods in Plant Pathology. Elsevier Scientific Pub. Co., NY. 312-314.

Nicholson, G.S. and R. Hammerschmidt.1992. Phenolic compound and their role in resistance. Ann. Rev. Phytopathol. 30: 369-389.

Niedz, R.P., H. Doostdar, T.G. McCollum, R.E. McDonald. and R.T Mayer. 1994. Plant defensive proteins and disease resistance in citrus. Proc. Fla. State Hort. Soc. 107: 79-82.

Oviasogie, P.O., D. Okoro, and C.L. Ndiokwere. 2009. Determination of total phenolic amount of some edible fruits and vegetables. Afr. J. Boiotechnol. 8(12): 2819-2820.

Pullaiah, N., G.J. Moses and P.S.R. Kumar. 1993. Biochemical nature of resistance in citrus to canker disease (*Xanthomonas compestris.* pv*. citri* (Hasse) Dye). J. Res. A.P.A.U. 21(3): 172-73.

Thimmaiah, S.R. 2004. Standard methods of Biochemical Analysis. Kalyani Publishers, New Dehli, India. pp. 54-55.