EVALUATION OF EXOTIC CITRUS ROOTSTOCKS AGAINST *FUSARIUM* SPP.

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Citrus is commercial fruit crop cultivated on a large scale in Pakistan. Citrus rootstocks play an import role in production and disease tolerance/resistance. *Fusarium* is one of the important devastating diseases of citrus. In present study twelve citrus rootstocks (11 exotic and 1 indigenous) were screened for their degree of susceptibility or resistance against *Fusarium*. These rootstocks were also studied biochemically to profile phenolic, total soluble sugars and protein content. Each rootstock showed different disease sensitivity index (%) when injected with different fungal specimens. Knorr Nucellar was highly susceptible to *Fusarium oxysporum* while Citrus sunki and Rough lemon were resistant against *Fusarium oxysporum*. In case of *Fusarium semitectum*, Knorr nucellar was highly susceptible but Rough lemon was moderately resistant. Citrus rootstocks X639 and Kirumakki were highly susceptible and moderately resistant, respectively, against *Fusarium* sp. Effective way of controlling the disease is by using resistant/tolerant rootstocks because *Fusarium* is a big hazard for citrus production.

Keywords: Rutaceae, rootstocks, tree vigor, citrus pathogens, citrus dieback, disease resistance, phenolic compounds, *Fusarium* sp.

INTRODUCTION

Citrus is a wide group of trees or shrubs belongs to the family *Rutaceae*. Citrus origin is believed to be the China, Southern Himalaya and North East of India (Gmitter *et al*., 1990). It is widely grown in sub-tropical and tropical climates, where the climatic regimes and soil are supportive for its growth and development. Due to its juice industry, ornamental value and importance in terms of human health, citrus production around the globe is increased. In 2017, Pakistan citrus fruit

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Commented [A6]: 5-7 keywords and must be other than appeared in title and abstract production was 1585090 tons and it is the 10th largest producer of citrus worldwide. Pakistan also exports about 305,627 tons of mandarins (Anonymous, 2017).

A citrus tree comprises two parts: 1) scion which is present above ground and 2) rootstock which is most important part of citrus tree and provides root system to the tree. There is no argument over the significance of citrus rootstocks for improved citrus production. Rootstock plays a significant role to regulate the performance and vigor of the tree. Effects of citrus production, precocity, maturity and tree size are attained through complex inter-relationship among root and canopy of plant. Rootstock directly affects uptake of nutrients and water from soil (Richardson *et al*., 2003).

Similarly, citrus is susceptible to several diseases caused by different pathogens (Mendonca *et al*., 2017). Among all diseases, *Fusarium* is one of the most devastating diseases affecting citrus. It is most important soil borne disease of citrus. Infection of *Fusarium* contains long-term symptoms. The symptoms produced by this disease are twigs dieback, leaf veins chlorosis, yellowing and dropping of foliage (Spina *et al*., 2008). Later this disease causes extensive root rot with production of phytotoxins (Strauss and Labuschagne, 1995). *Fusarium* wilt is produced by *Fusarium oxysporum* and is diverse in environment. *Fusarium semitectum* is a different species of *Fusarium* which is also a soil borne disease and usually exist in sub-tropical and temperate areas. This fungus produces contaminants such as deoxynivalenol and fumonisin. Optimal temperature for *Fusarium semitectum* growth is 24-25°C (Latiffah *et al*., 2009).

Disease resistance is one of the most important roles of rootstock. A natural system of defense exists in plants which needs to be discovered and utilized. Under stress plants develop line of defense which provides a complex response system against diseases for survival (Nafees *et al*., 2019a). This is the reason studies are now concentrating on the plants to overcome biotic and abiotic threats. Every rootstock has different concentration of biochemical compounds i.e. phenolics which varies in resistance against diseases. So, it is very essential to screen out different rootstocks against *Fusarium* so we can minimize the threat to citrus industry

MATERIALS AND METHODS

Present research was conducted to screen exotic citrus rootstocks against *Fusarium* disease. This experiment was carried out in Institute of Horticulture Sciences in association with Department of Plant Pathology and Department of Chemistry, University of Agriculture Faisalabad.

***Materials*:** Twelve citrus rootstocks were used in this experiment. Healthy and mature fruits of eleven exotic rootstocks, i.e. X639, Rich 6-16, Knor nucellar, *Poncirus trifoliata*, Citrus sunki, Srirampur nucellar, Kirumakki nucellar, Rangpur poona nucellar, Gabbuchinee, Clausena harmandiana, Papuan and one local commercial rootstock, i.e. Rough lemon were collected from Fruit Experimental Area, 32 square, Institute of Horticultural Sciences, University of Agriculture Faisalabad, Pakistan.

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Figure 1. Fruits of exotic citrus rootstocks.

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***Material preparation*:** Fruit was cut horizontally in two halves and seeds were extracted and washed. Healthy seeds were covered with masculine cloth and dipped for 10-15 minutes in stirring hot water of 52°C for uniform treatment of seeds to remove microbial contamination. Then, seeds were cooled and dipped in 10% NaOCl for 15-20 minutes. Seeds were then washed 3-4 times with distilled water (Usman *et al*., 2014) and sown on sterilized media (Sand: Silt: FYM; 1: 1: 1). Screening was madein greenhouse to evaluate susceptible or resistant rootstocks.

***Fungal specimen collection and conservation*:** Specimens were collected from Fungal Culture Bank of Pakistan, Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan (Table 1).

Table 1. Fusarium isolated from plant tissue.

FCBP Acc. No.	Name of fungi	Source of isolation	Preservation condition
FCBP-PTF-866	*Fusarium oxysporum*	Citrus root, Punjab University Housing colony, Lahore	4°C
FCBP-PTF-1189	*Fusarium semitectum*	*Zea mays* stored seeds. Lahore	4°C
FCBP-PTF-580.	*Fusarium sp.*	*Citrus limon* fruit, Lahore	4°C

Multiplication and inoculation of fungal culture**:** Pure fungal cultures were multiplied on nutrient agar. After multiplication, culture was inoculated on 13 months old rootstocks as 1 inch upper soil layer was removed from the pots and fungal culture was mixed with soil. Each fungal culture was inoculated on each rootstock to check their resistance or susceptibility.

Scale to test the level of sensitivity of disease against Fusarium**:** Disease severity index (%) was recorded according to scale described in Table 2. Calculation of disease index was made according to Croxal, (1952) on each rootstock using the formula;

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected leaves}}{\text{Total number of leaves}} \times 100$$

Table 2. Scale of disease severity index (%).

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Grades	Disease severity (%)	Response	Symbols
0	00-00	Immune	I
1	1-20	Resistant	R
3	21-40	Moderately Resistant	MR
5	41-60	Moderately susceptible	MS
7	61-80	Susceptible	S
9	81 and above	Highly susceptible	HS

Total phenolic contents (TPC)**:** TPC of diseased leaves was calculated by using Folin-Ciocalteu reagent method as reported by Ainsworth and Gillespie (2007). The absorbance was read at 765 nm and the amount of TPC was estimated by using standard curve for Gallic acid. The results were expressed as Gallic acid equivalent (GAE) per dry matter.

***Soluble protein contents*:** The soluble proteins of the fruit extracts were determined by the Bradford method (Bradford, 1976) and absorbance was taken at 595 nm.

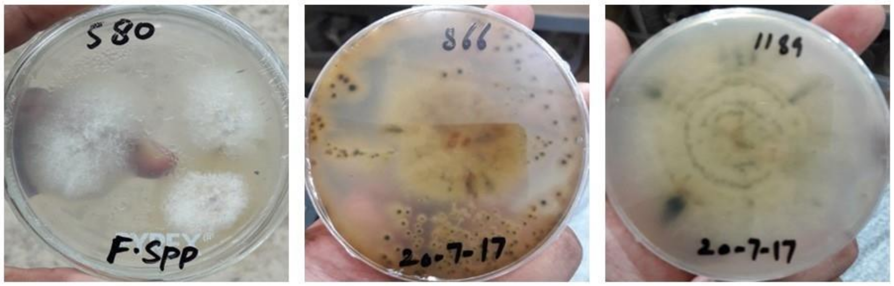
***Total sugars*:** TSS were determined by Anthrone reagent following Thimmaiah (2004). Briefly 8 mg of Anthron reagent was taken in 250 mL beaker and then 40mL H_2SO_4 was added to make reaction mixture. After preparing reaction solution, 1 mL from the above prepared solution was taken and mixed with 100 µl samples. Test tubes containing reaction mixture were kept in water bath for 1 hour. Samples were cooled and then absorbance was read at 630 nm.

***Leaf weight loss (%)*:** Fresh weight of leaves was recorded on weighing balance in grams (g). Then leaves were kept under shade for 2-3 days before putting it in incubator for 1 day at 62°C. The weight loss (%) was calculated by subtracting dry weight from fresh weight over fresh weight and then multiplying with 100.

***Statistical analysis*:** All analytical parameters were tested in triplicates and the calculated data was statistically analyzed by using CRD two factor factorial experiments and means were compared through analysis of variance technique (ANOVA) Tuckey test.

RESULTS

Resistance and susceptibility level were determined after 1 month of inoculation of fungal culture on plants (Fig. 1, 2 and 3). No pathogen was inoculated on control rootstocks and remained healthy. All rootstocks when evaluated against different *Fusarium* strains showed different level of disease incidence/ index.



Fusarium spp. *Fusarium oxysporum* *Fusarium semitectum*

Figure 2. Different *Fusarium* specimens.

Reaction of exotic citrus rootstocks against *Fusarium oxysporum*, *Fusarium semitectum* and *Fusarium* spp.:

***Disease sensitivity index*:** *Fusarium oxysporum* inoculum was applied artificially on twelve citrus rootstocks in greenhouse. High disease sensitivity was recorded in Knorr nucellar (100%), Srirampur nucellar (96.0%), Gabbuchinee (84.6%) and Kirumakki nucellar (93.3%) while Citrus sunki (3.7%) and Rough lemon (2.5%) were less susceptible to *Fusarium oxysporum*. In case of *Fusarium semitectum*, Rough lemon was moderately susceptible; however, Knorr nucellar (100%), Rangpur Poona nucellar (100%), Rich 16-6 (89.9%), Kirumakki nucellar (84.6%), Srirampur nucellar (100%), Clausena harmandiana (86.7%) and Gabbuchinee (100%) were highly susceptible. The effect of *Fusarium* spp. inoculum (applied artificially) revealed high disease sensitivity in Clausena harmandiana (81.3%), Gabbuchinee (92.3%), Rich 16-6 (93.3%), Rangpur poona nucellar (93.3%), Srirampur nucellar (100%), Rough lemon (90.0%), X639 (100.0%), Citrus sunki (100%) and Knorr nucellar (100%). Kirumakki nucellar (31.3%) was moderately resistant against *Fusarium* spp. as shown in Tables 3, 4 and 5.

***Total phenolic content (mg GAE/100 g FW)*:** Total phenolic content (TPC) showed negative linear trend with disease sensitivity index (%). The results showed that Rough lemon had high TPC with low disease sensitivity index. Low phenolic contents were recorded in Srirampur nucellar with high disease sensitivity index against the inoculum of *Fusarium oxysporum*. In case of *Fusarium semitectum*, high TPC was found in Rough lemon and low in Rich 16-6. While, the results of TPC against *Fusarium* spp. revealed that Kirumakki nucellar had high TPC with low disease sensitivity index and low phenolic contents in X639 with high disease sensitivity index.

***Protein determination (g/100 g FW)*:** There was a negative linear trend of total soluble protein contents with disease sensitivity index (%). The contents of protein were low in leaves of more susceptible rootstocks. So, maximum protein was found in Rough lemon with low disease index and low protein contents in Knorr nucellar with high disease sensitivity index against *Fusarium oxysporum*. In case of *Fusarium semitectum*, Rough lemon showed high protein content with low disease sensitivity index (%). Less protein contents were recorded in Rangpur poona nucellar with high disease sensitivity index. The results of inoculation with *Fusarium* spp. revealed that the protein contents in Kirumakki nucellar was high with low disease sensitivity index and low protein contents in Knorr nucellar with high disease sensitivity index.

***Total soluble sugars (mg/g)*:** A positive linear trend with disease sensitivity index (%) was recorded for total soluble sugars. Generally high sugars with high disease sensitivity index (%) was observed. Therefore, in case of *Fusarium oxysporum* and *Fusarium semitectum* maximum sugars were found in Gabbuchinee, with high disease sensitivity index. However, low TSS with low disease sensitivity index was recorded in Citrus sunki and Rough lemon. The inoculation of *Fusarium* spp. resulted high TSS with high disease sensitivity index in rootstock Srirampur nucellar.

***Weight loss (%)*:** Weight loss (%) had positive linear trend with disease sensitivity index (%). It means that if there is high disease sensitivity index then there will be high weight loss in plants. So, the highest weight loss with high disease sensitivity index was recorded in Knorr nucellar and the lowest in Rough lemon with low disease sensitivity index when inoculated with *Fusarium oxysporum*. In case of *Fusarium semitectum*, the highest weight loss with high disease sensitivity index was recorded in Rangpur poona nucellar, while the lowest was recorded in Rough lemon. Moreover, *Fusarium* spp. inoculum revealed high weight loss with high disease index in X639.

Table 3. Citrus rootstocks as affected by *Fusarium oxysporum*.

Rootstocks	Disease index (%)	TPC (mg GAE /100 g)	Protein (mg/100g)	Total soluble sugars (mg/ 100 g	Wt. loss (%)
X639	51.7	122.79 hijk	6.180 efg	813.3 cde	39.557 fghi
Rich 16-6	25.7	319.15 c	7.000 defg	355.2 ij	23.820 ghi
Knorr nucellar	100.0	93.67 jk	3.600 g	879.0 bcd	32.680 bcdef
Poncirus trifoliata	34.8	452.47 b	6.200 efg	632.4 fg	46.907 efghi
Citrus sunki	3.7	539.34 a	7.240 defg	224.3 j	27.753 hi
Srirampur nucellar	96.0	75.36 k	4.337 g	902.9 bc	32.660 cdefg
Kirumakki nucellar	93.3	84.93 k	3.850 g	959.1 ab	28.720 bcde
Rangpur poona nucellar	50.0	205.01 efg	7.107 defg	448.1 hi	21.380 bcdef
Gabbuchinee	84.6	201.40 efg	4.517 g	971.0 ab	34.727 cdefg
Clausena harmandiana	80.0	86.44 k	3.647 g	567.1 gh	36.033 bcdef
Papuan	50.0	121.94 ijk	5.977 fg	556.2 gh	41.250 cdefg
Rough lemon	2.5	508.15 ab	9.827 bcdef	255.2 j	20.277 i

Table 4. Citrus rootstocks as affected by *Fusarium semitectum*.

Rootstocks	Disease index (%)	TPC (mg GAE /100 g)	Protein (mg/100g)	Total soluble sugars (mg/ 100 g	Wt. loss (%)
X639	73.1	191.56 de	6.080 fghij	829.8 cde	51.677 cdefg

Rich 16-6	86.7	175.97 def	7.590 efghij	737.1 ef	49.530 defg
Knorr nucellar	100.0	97.93 h	4.027 ij	878.6 bcde	44.747 efg
Poncirus trifoliata	78.9	207.48 d	5.480 ghij	795.2 def	55.733 bcdef
Citrus sunki	70.0	274.89 c	5.933 ghij	915.2 abcde	49.060 defg
Srirampur nucellar	100.0	136.79 fgh	6.533 fghij	976.2 abcde	57.160 bcdef
Kirumakki nucellar	86.7	207.47 d	4.700 hij	881.0 bcde	58.033 bcdef
Rangpur poona nucellar	100.0	156.14 efg	2.730 j	787.6 def	62.727 bcd
Gabbuchinee	100.0	116.63 gh	5.573 ghij	963.3 abcde	51.457 cdefg
Clausena harmandiana	86.7	219.08 d	7.570 efghij	860.0 cde	43.187 fg
Papuan	50.0	333.09 ab	13.423 abcd	488.1 fg	37.227 gh
Rough lemon	25.0	303.83 bc	15.000 abc	349.5 g	22.453 h

Table 5. Citrus rootstocks as affected by *Fusarium* spp.

Rootstocks	**Disease index (%)**	**TPC (mg GAE /100 g)**	**Protein (mg/100g)**	**Total soluble sugars (mg/ 100 g**	**Wt. loss (%)**
X639	100	114.34 g	4.507 h	973.8 bcde	57.317 abcde
Rich 16-6	93.3	134.03 g	7.860 defgh	805.7 efgh	41.130 abcde
Knorr nucellar	100	159.77 fg	4.590 h	760.9 efgh	44.557 abcde
Poncirus trifoliata	62.5	348.99 abc	11.170 bcdefg	648.6 gh	35.640 de
Citrus sunki	100	187.29 efg	6.810 efgh	879.0 cdef	49.523 abcde
Srirampur nucellar	100	178.60 efg	5.640 fgh	1107.6 b	54.370 abcde
Kirumakki nucellar	31.3	391.78 ab	14.380 bc	370.9 i	21.567 e
Rangpur poona nucellar	93.3	134.59 g	8.473 cdefgh	793.7 efgh	48.607 abcde

Gabbuchinee	92.3	178.30 efg	8.720 cdefgh	760.9 efgh	39.900 bcde
Clausena harmandiana	81.3	147.21 g	5.370 gh	745.2 fgh	47.393 abcde
Papuan	76.5	183.41 efg	9.670 bcdefgh	598.6 h	38.630 cde
Rough lemon	90	121.16 g	6.530 efgh	848.6 defg	45.403 abcde

DISCUSSION

Fusarium is a big hazard for citrus production. Our result confirms the finding of (Tiaz and Zeiger, 2002) in which they assessed that secondary metabolites can be produced in citrus plants which also contains phenolic. Phenolic are chemically varied compound chiefly tannins, flavonoids and lignin that play dynamic role in resistance from disease.

Protein contents also have a role in managing disease of *Fusarium* in plants because proteins limit the reproduction of bacteria, viruses and fungi. Proteins also bound pathogen transportation in healthy tissues. These proteins are named as pathogenesis connected proteins. Plants cannot run from the pathogen's epidemics, so they mature a fight mechanism to defend themselves against microbial diseases (Dangal and Jones, 2001; Nafees *et al*., 2019b). It has been reported that many disease resistant plants show higher quantity of protein content. Although some proteins are receptor of pathogen but most of the proteins show negative trend with disease incidence. If the disease attack in plant is high then there will be less protein concentration in plants (Martin, 2003). New knowledges rising from the proteomics and genomics uprising will greatly enlarge our aptitude to examine the role of R proteins in plant disease resistance.

Our results showed that sugar contents were high in plants with high disease susceptibility. This agrees with previous findings of other research workers (Nath *et al*., 2015) who determined total sugar in infected and uninfected banana fruits and foliage when affected by fruit rot disease.

It was observed that plants weight loss (%) was maximum with increase in disease susceptibility. James, (1974) studied the weight losses in pathogen attacked plants and stated that there was a great decrease in plant foliage weight. When fungal disease attacks to plant then plant wilts, which reduce the weight in plant foliage. In this experiment we screened different plants foliage against different plant fungal diseases to estimate the weight loss (%) and concluded that when disease attacks to plant foliage there is a loss of weight in plants.

Conclusion**:** Excellent and resistant rootstock use can reduce and prevent the losses of root rot and wilt caused by *Fusarium*. In this study Citrus sunki and Rough lemon showed resistance against *Fusarium oxysporum*. Moderate resistance was revealed by Rough lemon against *Fusarium semitectum* and Kirumakki nucellar against *Fusarium* sp. *Fusarium* is a big threat to citrus industry, hence there is a need to use resistant rootstocks to improve the longevity and productivity citrus groves.

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REFERENCES

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Ainsworth, A.A. and K.M. Gillespie. 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nature protocols 4:875-877.

Anonymous. 2017. Food and Agriculture Organization of the United Nations. Available online at http://faostat.org

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

Croxal, H.E., D.C. Gwyne and J.E. E. Jekins. 1952. The rapid assesment of apple scab on leaves. Plant Pathol. 1:39-41.

Croxal, H.E., D.C. Gwyne and J.E.E. Jekins. 1952. The rapid assessment of apple scab on leaves. Plant Pathol. 1:39-41.

Dangal, J.L. and J.D. Jones. 2001. Plant pathogens and integrated defense response to infection. Nature 411:826-833.

Gmitter, F.G. and X. Hu .1990. The possible role of Yunnan, China, in the origin of contemporary *Citrus* species (Rutaceae). Econ. Bot. 44:267- 277.

James, W.C. 1974. Assessment of plant diseases and losses. Ann. Rev. Phytopath. 12:27-48.

Latiffah, Z. 2009. Diversity of *Fusarium* species in cultivated soils in Penang. Malaysian J. Microbiol. 3:27-30.

Martin, G.B., A.J. Bogdanove and G. Sessa. 2003. Understanding the functions of plant disease resistance proteins. Ann. Rev. Plant Biol. 54:23-61.

Mendonca, L.B.P., L. Zambolim and J.L. Badel. 2017. Bacterial citrus diseases: major threats and recent progress. J. Bacteriol. Mycol. 5 340-350.

Nafees, M., M.A. Bukhari, M.N. Aslam, I. Ahmad, M. Ahsan and M.A. Anjum. 2019a. Present status and future prospects of endangered Salvadora species: A review. J. Glob. Innov. Agric. Soc. Sci. 7:39-46.

Nafees, M., M.J. Jaskani, A. Sajjad, M.N. Aslam, I. Ahmad and M.M. Abbas. 2019b. Tree modeling in horticultural crops: A review**.** J. Glob. Innov. Agric. Soc. Sci. 7:87-97.

Nath, K., K.U. Solanky, M.K. Mahatma and S.R. Madhubala. 2015. Role of total soluble sugar, phenols and defense related enzymes in relation to banana fruit rot by *Lasiodiplodia theobromae* [(Path.) Griff. and Maubl.] during ripening. J. Plant Pathol. Microbiol. 6:1-6.

Richardson, A.E., J.P. Lynch and P.R. Ryan. 2003. Water and nutrients uptake in plants. Plant Soil 349:121-156

Spina, S., V. Coco, A. Gentile, A. Catara and G. Cirvilleri. 2008. Association of *Fusarium solani* with rolABC and wild type Troyer citrange. J. Plant Pathol. 90:479-486.

Strauss, J. and N. Lauschange. 1995. Pathogenicity of *Fusarium solani* isolates on citrus roots and evaluation of different inoculum types. Appl. Plant Sci. 9:48-52.

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

Usman, M., M.H. Shah, A. Badar, B. Fatima, M. Sabirr and Q. Zaman. 2014. Media sterilization and coco coir enhanced growth of rough lemon nursery. Pak. J. Agri. Sci. 51:615-623.