African Journal of Biotechnology Vol. 11(9), pp. 2199-2203, 31 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2125
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Toxin production by *Fusarium solani* from declining citrus plants and its management

Abdul Rehman[1]*, Atta Rehman U.[1], Nazir Javed[1], Aman Ullah Malik[2] and Saira Mehboob[3]

[1]Department of Plant pathology, University of Agriculture Faisalabad, Pakistan.
[2]Institute of Horticultural Sciences, University of Agriculture Faisalabad, Pakistan.
[3]Institute of Plant Pathology, Ayyub Agricultural Institute Faisalabad, Pakistan.

Accepted 25 October, 2011

The core purpose of this current research was to meticulously survey four tehsils of Sargodha district and to probe the present status of citrus decline in infected citrus orchards. The utmost fungi (39.52%) were secluded from the roots followed by the soil (38.86%). The highest *Fusarium* sp. followed by *Aspergillus*, *Phytophthora, Pythium*, *Penicillium* and *Alternaria* species were remote from the collected samples of roots and soil from the four tehsils of Sargodha district of Pakistan. The maximum *Fusarium* sp. was isolated from the roots of declining citrus trees from tehsil Bhalwal (68.57%) followed by Kot Momin (65.87%), Sillanwali (55.87%) and Sargodha (50.32%). Toxin studies were also carried out using thin layer chromatography which revealed that *F. solani* produces toxins (anhydrofusarubin) which may cause decline in citrus. *In vitro* effect of fungicides on the mycelial growth rate of *Fusarium solani* exposed aliete to be more effective at 50 and 100 ppm, respectively. Ridomil Gold and Dithane M-45 showed same effect while Deconil showed least effect.

Key Words: Citrus, citrus decline, *Fusarium solani*, toxin, Anhydrofusarubin, aliete.

INTRODUCTION

Citrus is grown throughout the world including Pakistan, and is among the most valuable fruit crop in international trade. Citrus occupies a renowned position in fruit industry of the world. It is the world recognized agricultural commercial fruit crop and is grown in more than 125 countries falling in a belt within 35° latitude north or south of the equator (Duncan and Cohn, 1990). It has high nutritional value and is a prosperous source of vitamin C in conjunction with sugar, organic acids, amino acids and minerals resembling calcium and magnesium in an ample amount (Niaz et al., 2004).

In Pakistan, citrus stands first among all the cultivated fruits with an area of 853 hectares and total production of 7178 tones during 2007 to 2008. In Pakistan, it is grown all over the country but Punjab ranks first in terms of area and production owing to highly suitable climate for citrus production, in exacting, the Sargodha district. It contributes about 65% of the total fruit production in Pakistan (Anonymous, 2008). The foremost citrus varieties grown commercially are mandarins, sweet orange, grape fruit, lemon and lime.

Citrus fruit production in Pakistan has been abridged steadily as compared to other citrus growing countries as a consequence of many pre and post harvest problems. The ailments and pests allied with citrus are among the most serious dilemmas causing stern losses in both quality and quantity. A number of pathogens including fungi, bacteria, nematodes and virus cause a number of diseases in citrus.

There are many constraints to citrus production around the world and citrus decline and replanting problems have been recognized in several regions of the world. Martin and Joseph (1946) mentioned that soil fungi were partly responsible for citrus decline in the United States of America.

Fusarium species persuade symptoms on roots principally feeder roots; the characteristic symptoms are deprived growth of tree, leaf drooping and yellowing, wilting of plant, root rottening and shredding of bark of roots

*Corresponding author. E-mail: arb041@gmail.com. Tel: +92 333 8991639. Fax: +92 41 240 9303.

particularly feeder roots. Fungus penetrates in the roots, instigating rottening, and then move to xylem vessels, causing plant wilting and ultimately death of plant. If environmental factors are inauspicious and plant is under stress, it may cause the death of the tree within a few days (Booth, 1973). Renato et al. (2003) reported that citrus decline was first time reported in Brazil in 1999, since then it is becoming a serious problem with passing days in citrus growing areas.

Fusarium solani is associated with two types of syndrome namely dry root rot and chronic feeder root rot that cause slow decline of citrus (Bender et al., 1982).

The present studies was undertaken to investigate the cause of citrus decline, toxins study and evaluate some fungicides against the isolated fungus *in vitro* conditions.

MATERIALS AND METHODS

Survey

A survey of best citrus mandarin producing area of the Punjab *viz.,* Sargodha district was carried out to study the severity of citrus decline. The roots and soil samples were collected for the isolation of the associated fungus. The samples were collected from different citrus orchards and processed separately. Isolations were made on potato dextrose agar (PDA) medium.

Isolation of fungal pathogens

The infected samples were cut into 1 to 2 cm length and the surface was sterilized with 70% ethyl alcohol for 1 to 2 min. Sterilized pieces were rinsed twice with distilled water and transferred on to sterilized filter paper in Petri plates for drying. Then, pieces were plated on Petri plates containing PDA media, using untainted forceps. For cutting and transferring these pieces, scissors and needles needed for use were sterilized by dipping in methylated spirit and flaming several times. All Petri plates were incubated at 27°C for 5 to 7 days and observed on daily basis (Roger and Dean, 2005). The fungi which colonized on these pieces were purified and identified on the basis of characters (Booth, 1977; Neergaard, 1979). The frequency of each isolated fungus from each part in the cultured pieces was calculated by using the following formula:

$$\text{Colonization \%} = \frac{\text{Number of pieces colonized by a pathogen}}{\text{Total number of pieces}} \times 100$$

For the isolation of soil fungi, again two methods were applied. First, soil was placed directly on PDA medium in sterilized Petri plates which were sealed and incubated at 25°C for 7 days. The out growing colonies were counted and purified on agar slants for further studies (Lester et al., 2008).

Dilution plate technique

Modified soil dilution plate technique was used for isolating the fungi from infested rhizosphere soil of citrus (Lester et al., 2008). In this technique, the air dried soil samples were ground with mortar and pestle and mixed thoroughly. 10 g sub sample of rhizosphere soil of citrus was transferred to 100 ml of sterile 0.01% water in a bottle, to give a dilution of 1:10. 10 ml of suspension was transferred to a second bottle containing 90 ml of water to give a dilution of 1:100 and mixed well. This step was repeated to give 1:1000 dilutions. 1 ml of soil suspension was dispersed across the medium in a 90 mm diameter Petri dish, incubated at 25°C with 12 h alternate periods of light and darkness for 7 days and were observed on daily basis. The out growing colonies of fungal pathogens were purified on PDA slants and were identified under microscope (Ellis, 1971).

Toxin studies

For this purpose, potato dextrose (PD) broth was prepared and autoclaved at 121°C (15 psi) then inoculated with the test fungi after cooling. Inoculated flasks were incubated at an optimum temperature of 25°C. Mycelia mat of fungus was filtered off through muslin cloth held over a glass funnel after 15 days of incubation. The filtrate was kept in small bottles at freezing temperatures for further toxin studies.

Isolation and purification of toxin from culture filtrate

Culture filtrate was treated with ethyl acetate (EA) followed by ethyl ether which evaporated and the residue was extracted with petroleum ether. The latter was evaporated to obtain the crystals of the toxins. The pH of the culture filtrate was adjusted with 5 N HCl to 4.0 and extracted 3 times with EA using 1.5 L of the solvent for every extraction and allowed at least 30 min for each extraction. EA extracts were pooled and volume reduced to 100 ml on a rotary evaporator or hot water bath. 50 ml of water was added; pH adjusted to 8.0 with NaOH and extracted with 150 ml of ethyl ether in Soxhlet extractor for about 24 h. Ether extract was dried for about 2 h with 20 g of anhydrous sodium sulphate after which the ether was distilled off. The residues were extracted with 100 ml of hot petroleum (b. p. 60 to 90°C) using a reflux condenser for five times and changing the solvent at 30 min interval. Combined petroleum ether extracts were evaporated on a hot water bath (crystals of the toxin appeared) and residue was dissolved in 1 to 2 ml of ethanol (Thimmaiah, 2004).

Thin layer chromatography (TLC)

TLC was carried out by using butanol: acetic acid: water (75: 15: 10 v/v) to detect the mycotoxins produced by the fungi. The chromoplates were removed after 90 min when the solvent reached 3.5 to 4.0 cm from the upper edge of the chromatoplates. They were dried mechanically and chromo-plates were read by observing the chromatographic spots under ultraviolet light of 254 and 356 nm length (Baker et al., 1981).

Management of pathogenic fungi

To test the *in vitro* efficacy of fungicides; Aliete, Ridomil Gold, Dithane M-45, Score and Deconil at 20, 50 and 100 ppm concentrations were checked using poison food technique (Nene and Thakliyar, 1979). Each fungicide was mixed separately in autoclaved PDA medium to obtain required concentration that is, 20, 50 and 100 ppm. 20 ml of poisoned PDA was poured into each sterilized plate and allowed to solidify; PDA medium without fungicides served as control. After solidification of medium, 3 mm agar plugs of the test fungus were transferred in the center of the PDA plates; each treatment was replicated thrice. Petri plates were incubated at 25 ± 2°C. The data were recorded in term of radial growth rate after 8 days. The effective fungicide was determined by

Table 1. Frequency of fungi isolated from citrus tree roots from different orchards of Sargodha district.

Tehsil	Species			Mean
	Fusarium solani	*Alternaria alternata*	*Fusarium semitectum*	
Kot Momin	65.87 ± 1.52	25.11 ± 3.58	25.33 ± 3.53	38.77 ± 6.94
Bhalwal	68.57 ± 4.36	24.45 ± 2.22	25.56 ± 2.94	39.52 ± 7.45
Sargodha	50.32 ± 7.02	29.76 ± 2.68	28.22 ± 4.22	36.10 ± 4.35
Sillanwali	55.87 ± 5.11	27.24 ± 2.43	29.67 ± 5.18	37.59 ± 5.09
Mean	60.16 ± 3.06[a]	26.64 ± 1.34[b]	27.20 ± 1.81[b]	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05).

Table 2. Frequency of fungi isolated from the rhizosphere soil (Direct inoculation method) of citrus trees from different orchards of Sargodha district.

Tehsil	Species			Mean
	Fusarium solani	*Phytophthora* sp.	*Aspergillus* sp.	
Kot Momin	60.95 ± 0.95	26.11 ± 2.00	26.22 ± 1.18	37.76 ± 5.84
Bhalwal	58.10 ± 7.80	27.11 ± 0.44	27.70 ± 1.93	37.64 ± 5.62
Sargodha	59.36 ± 6.83	24.89 ± 2.48	32.33 ± 1.45	38.86 ± 5.66
Sillanwali	52.38 ± 4.15	23.24 ± 1.69	31.67 ± 2.03	35.76 ± 4.56
Mean	57.70 ± 2.58[a]	25.34 ± 0.89[b]	29.48 ± 1.06[b]	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05).

calculating colony diameter on daily basis for 8 days and to develop the most effective dose rate (Nene and Thakliyar, 1979).

RESULTS AND DISCUSSION

Survey of citrus orchards

In order to determine the disease incidence, an extensive survey of Sargodha district was conducted. The maximum fungi (39.52%) were isolated from the roots followed by the soil (38.86%). The maximum *F. solani* isolated from the collected samples of roots and soil where from the four tehsils of Sargodha district.

Isolation of fungal pathogens from roots

The analysis of variance of data was used to determine the frequency of fungal species isolated from roots of citrus trees growing in Kot Momin, Bhalwal, Sargodha and Sillanwali of Sargodha district. The maximum percentage of *F. solani* was isolated from the roots of declining citrus trees from the tehsil Bhalwal (68.57%) followed by Kot Momin (65.87%), Sillanwali (55.87%) and Sargodha (50.32%), (Table1).

Nemec et al. (1980) isolated *F. solani* from citrus roots and confirmed its pathogenecity and reported that it was associated with feeder roots and caused wilting of citrus roots. Preliminary research on the roots of the declining trees indicated the presence of some pathogenic fungi. The presence of these fungi suggests that they contribute to decline and citrus replant problems (Hassan et al., 1989).

Isolation of fungal pathogens from soil

Direct inoculation method

Mean value of data on the frequency of fungal species recovered from the soil of declining citrus orchards in Kot Momin, Bhalwal, Sargodha and Sillanwali of Sargodha district is given in Table 2.

The maximum frequency of *F. solani* was isolated from the soil of declining citrus orchards from the tehsil Kot Momin (60.90%) followed by the Sargodha (59.36%), Bhalwal (58.10%), and Sillanwali (52.38%). *Aspergillus* sp. was isolated from the soil samples of citrus orchards as 32.33% from tehsil Sargodha, 31.67% from Sillanwali, 27.70% from Bhalwal and 26.22% from Kot Momin. *Phytophthora* was also isolated from the soil samples through direct inoculation method as 27.11, 26.11, 24.89 and 23.24% from tehsil Bhalwal, Kot Momin, Sargodha and Sillanwali, respectively.

Suit et al. (1953) and Sherbakoff (1953) found that several *Fusarium* species including *F. solani* and *Fusarium oxysporum* are common inhabitants of Florida citrus soil and are often found in the roots of healthy as well as diseased citrus trees.

Table 3. Frequency of fungi isolated from the rhizosphere soil (dilution plate technique) of citrus trees from different orchards of Sargodha district.

Tehsil	Species			Mean
	Fusarium solani	*Pythium*	*Penicillium*	
Kot Momin	5.83 ± 0.19	4.55 ± 0.15	5.55 ± 0.21	5.31 ± 0.22[b]
Bhalwal	6.24 ± 0.19	5.07 ± 0.19	6.57 ± 0.38	5.96 ± 0.26[a]
Sargodha	5.93 ± 0.46	5.26 ± 0.21	6.26 ± 0.35	5.82 ± 0.23[a]
Sillanwali	6.19 ± 0.35	4.86 ± 0.18	5.95 ± 0.17	5.67 ± 0.24[ab]
Mean	6.05 ± 0.15[a]	4.94 ± 0.11[b]	6.08 ± 0.17[b]	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.*05*).

Dilution plate technique

The analysis of variance of data on the frequency of fungal species recovered from the soil of declining citrus orchards through dilution plate technique is given in Table 3.

The maximum *F. solani* was isolated from the soil of declining citrus orchards from the tehsil Bhalwal (6.24%) followed by the Sillanwali (6.19%), Sargodha (5.93%) and Kot Momin (5.83%). *Penicillium* sp. was isolated from the soil samples as 6.57, 6.26, 5.95 and 5.55% from Tehsil Bhalwal, Sargodha, Sillanwali and Kot momin, respectively while *Pythium* sp. was isolated as 5.26% from tehsil Sargodha, 5.07% from Bhalwal, 4.86% from Sillanwali and 4.55% from Kot Momin.

Rensburg et al. (1996) recovered a total of 42 isolates from soil of citrus orchard, and isolated *F. solani* (84%), *Fusarium semitectum* (7%), *Fusarium equiseti* (7%), and *F. oxysporum* (2%) and they found that *F. solani* is pathogenic to citrus by producing different toxins such as isomarticin and napthozarines. This study shows that *Fusarium* populations are diverse and may act as a potential inoculum to infect many agricultural crops.

Toxin studies

Effect of toxins produce by the F. solani on young citrus seedlings

To study the effect of toxins produced in cultural filtrate of *F. solani*, young citrus seedlings were placed in cultural filtrates in a test tube whereas test tube having PD broth without *F. solani* served as check. After 24 h, the effect of toxin was observed on seedlings in culture filtrate of *F. solani,* which showed wilting and withering whereas the seedlings remained as such which was placed in PD broth only. In pot test, infection of citrus seedlings with *F. solani* caused growth reductions and chlorosis (Nemec, 1978). The withering and wilting of seedlings in culture filtrate of *F. solani* indicates the presence of certain toxin.

Thin layer chromatography

TLC was carried out using butanol-acetic acid-water to make it probable to perceive the mycotoxins produced by *F. solani.* The chromo plates were detached after 90 min or when the solvent reached 2.5 cm from the upper edges of the chromo plates. Reading of the chromo plates was carried out by observing chromatographic spots from the discs containing the microorganism and their stand-ard was carried out under ultraviolet rays with 254 and 356 nm. The Rf value of chromatographic spots was observed to be 0.72 and 0.65 cm and the color was purple and yellow respectively from the culture filtrate of *F. solani*. This shows that *F. solani* from roots of citrus trees has the potential to produce phytotoxins. Baker et al. (1981) illustrated that the anhydrofusarubin toxin was produced by the *F. solani* which has the Rf value of the chromatographic spot 0.74 cm with purple color and 0.68 with yellow color though the Rf value in our study was a little bit different, and may be due to contamination. Kern and Naef-Roth (1965) exemplify that some isolates were not extremely toxic; strains of *F. solani* were known to vary widely in toxin production.

Efficacy of fungicides

The efficacy of the test fungicides in reducing the mycelial growth of *F. solani* assorted is given in Table 4. Aliete at 100 ppm entirely suppressed mycelial growth of *F. solani*. Ridomil gold at 50 and 100 ppm abridged the growth rate of *F. solani* by 65.60 and 66.89%, respectively. Dithane M-45 at 50 and 100 ppm also decreased the growth rate by 59.63 and 68.04%, respectively while Score reduced the growth rate at 50 and 100 ppm by 51.83 and 58.90%, respectively. However, *F. solani* was tolerant to Deconil, showing faster mycelial growth rate (4.33 and 4.17 mm day^{-1}) at 50 and 100 ppm, respectively. Some other fungicides such as Vitavax, Dithane M-45, Bavistin and Benlate were also known to have significant suppressive effect on the growth of *F. solani* (Ahmad *et al.,* 1996). Among the three fungicides evaluated against *F. solani in vitro*, Benomyl was found to be highly effective causing a significant reduction in mycelial growth of the test fungus even in very low concentration of 10 ppm. Ridomil was effective in higher concen-tration (Bajwa and Javaid, 2007).

Table 4. Effect of fungicides at different concentrations levels on the mycelial growth (mm day^{-1}) of *Fusarium solani* on potato dextrose agar at 25 ±1°C.

Treatment	20 ppm		50 ppm		100 ppm	
	Colony diameter	% Decrease over control	Colony diameter	% Decrease over control	Colony diameter	% Decrease over control
Aliete	1.75^{e}	75.81	1.25^{e}	82.80	0.17^{d}	97.72
Ridomil Gold	3.33^{cd}	53.92	2.50^{d}	65.60	2.42^{c}	66.89
Dithane M-45	3.17^{d}	56.22	2.93^{cd}	59.63	2.33^{c}	68.04
Score	3.83^{c}	47.00	3.50^{bc}	51.83	3.00^{c}	58.90
Deconil	4.50^{b}	37.79	4.33^{b}	40.37	4.17^{b}	42.92
Control	7.23^{a}		7.27^{a}		7.30^{a}	
LSD	0.571		0.882		0.684	
CV%	8.07		13.67		11.93	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.*05*).

Conclusion

Conclusively, soil borne fungi have a very important role in this malady which seems to produce toxin which is very important in citrus decline, and can be managed by applying certain chemicals covered in this paper.

REFERENCES

Anonymous (2008). Agricultural Statistics of Pakistan. Ministry of Food, Agriculture and Livestock (Economic Wing), Islamabad, Pakistan.

Ahmad M, Khan MA, Ahmad F, Khan SM (1996). Effectiveness of some fungicides on the colony growth of *Fusarium oxysporum* and *F. solani* associated with potato wilt. Pak. J. Phytopathol. 8: 159-161.

Booth C (1973). Gibberella zeae (*Fusarium graminearum*). CMI Descriptions of Pathogenic Fungi and Bacteria. p. 384.

Booth C (1977). The Genus Fusarium CMI, Kew, Survey, England.

Baker RA, Tatum JH, Nemec S (1981). Toxin production by *Fusarium solani* from fibrous roots of blight-diseased citrus. Phytopathology, 71: 951-954.

Bender GS, Menge JA, Ohr HO, Burns RM (1982). Dry root rot of citrus. It's meaning for the grower. *Citrograph*. 67: 249-254.

Bajwa R, Javaid A (2007). Integrated Disease Management to Control Shisham (*Dalbergia sissoo* roxb.*)* Decline in Pakistan. Pak. J. Bot. 39(7). 2651-2656.

Duncan LW, Cohn E (1990). "Nematode parasites of citrus", *In*: R. Luc, R. A. Sikora, and J. Bridge (eds.), Plant Parasitic Nematodes in Sub-tropical and Tropical Agriculture, CAB Int. pp. 321-346.

Ellis MB (1971). More *Dematiaceous Hyphomycetes*. CMI, Kew, Survey, England. p. 507.

Hassan MS, El-Behadli AH, Alsaadawi IS (1989). Citrus replant problem in Iraq. Plant Soil. 116: 151-155.

Kern,H, Naef-Roth S (1965). Zwei neue, durch martiella- Fusarien gebildete Naphthazarin derivate. *Phytopathol*. Z. 60: 316-324.

Lester WB, Timothy EK, Len T, Bhien TP (2008). Diagnostic manual for plant diseases in Vietnam. Australian Center for Agriculture Research in Canberra.

Martin JP, Joseph H (1946). Some observation on fungus flora of California citrus soils. Calif. Citrograph. 33: 198-200.

Neergaard P (1979). Seed Pathology, The McMillan Press Ltd. London. 1: p. 240.

Nemec S (1978). Symptomatology and histopathology of fibrous roots of rough lemon (citrus limon) infected with *Fusarium solani.* Mycopathologia, 63(1): 35-40.

Nemec S, Baker R, Burnett H (1980). Pathogenicity of *Fusarium solani* to citrus roots and its possible role in blight etiology. *In*. Proc. Florida State Hort. Soc. 93: 36-41.

Nene HL, Thakliyar PN (1979). Fungicides in plant disease control. Oxford and IBH Publishing company, New Delhi, p. 507.

Niaz AC, Maken MN, Malik SA (2004). Native home, historical background and importance of citrus fruits in Pakistan. *In:* Proc. 1st Int. Conf. on Citriculture. Uni. Agric., Faisalabad. 28-29 April. pp. 48-56.

Renato BB, Filho AB, Amorim L, Gimens-Fernandes, Gottwald TR, Bove JM. (2003). Spatial and temporal analysis of citrus sudden death as a tool to generate hypothesis concerning its etiologgy. Epidemiology, 93(4): 502-512.

Rensburg JCV, Labuschagne N, Nemec S (1996). Effect of napthazarin toxins produced by *Fusarium solani* on three citrus rootstocks in a hydroponic system. *In:* Proc. Int. Soc. Citriculture. VIII Int. Citrus Con. Sun City, pp. 427-430.

Roger S, Dean B (2005). Management of Plant pathogenic collection. Deapartment of Agriculture, Fisheries and Forestery, Australial Government.

Sherbakoff CD (1953) *Fusariam* associated with citrus feeder roots in Florida. Phytopathol. 43: 395-397.

Suit RFEP, DuCharme, Ford HW, Brooks TL (1953). Nature, causes and control of citrus decline. Ann. Rept. Fla. Exp. Sta. 1953: 188-190.

Thimmaiah SK (2004). Standard methods for Biochemical Analysis. Kalyani Pub. New Delhi.. 2nd ed, pp. 326-334.