Assessment of the potential for disease development of *colletotrichum spp.* in different species of citrus fruits.

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Abstract: Citrus is widely grown and most important fruit all over the world and Pakistan. At current, Pakistan stands among the top 11 citrus producing countries of the world. The industry of citrus is one of the major fruit industries all over the world; hence knowledge about pathogens associated with citrus crop is very essential. A lot of factors are responsible for its low production in the country including various pre and post-harvest diseases, citrus wither-tip and citrus anthracnose is one of them. The research was conducted for molecular diversity analysis of *Colletotrichum spp.* and the assessment of the potential for disease development of *Colletotrichum spp.* in different species of citrus fruits. For this purpose first of all, survey was done at three different places UAF, AARI and Khanuwana for collection of diseased samples. Disease incidence was recorded at Khanuwana (75%), AARI (60%) and UAF (50%). The infected citrus trees showed the branches and twigs appear to be scorched by fire. Drying of twigs occur from top to down word. Dry twigs are ash colored, while affected fruit show tear stain symptoms. Pathogenicity test was done on four different varieties of citrus (Kinnow, Musami, Shakri and Red Blood) to confirm Koch's postulates. Percent diseases index (PDI) was measured for the assessment of diseases development of *Colletotrichum spp.* After data recording Musami was found with maximum PDI while Kinnow was observed with least PDI. Morphological studies of the fungus were done on artificial PDA medium to study its salient features. Initially colony growth was found whitish to light gray in color as the culture becomes aged it turns into dark gray in color. Spores of *C. gloeosporioides* were observed typically elongated, hyaline, one celled with obtuse ends and non septate ovoid to oblong, 10-15µm in length and 5-7 µm in width. Conidiophores were erect, simple, and elongated. For molecular diversity analysis, genomic DNA isolation was done by following modified CTAB method. Seven different isolates of *Colletotrichum spp.* were isolated and their confirmation was done through gel-electrophoresis. RFLP was carried out to check the genetic diversity amongst different isolates of *Colletotrichum spp.* The RFLP results revealed that same type of *Colletotrichum spp.* is prevailing in citrus orchards of district Faisalabad.

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Introduction

Pakistan is among the countries being blessed with diverse climatic conditions by nature, which are favorable for production of temperate, subtropical and tropical fruits. Presently, worlds fruit production is estimated about 33519.3250 million tons cultivated on an area of 4874.77 (000) ha (Anonymous, 2016). Citrus cultivation is done on large scale and ranked at number one in production (121 MT) and area (1361.4 thousand ha) worldwide (FAOSTAT, 2015). Like in the world, citrus stands first in the production (2.39 MT) and area (195 thousand ha) in Pakistan, and it contributes 25% to GDP (Anonymous, 2015). About 95% of citrus fruits are produced and in Punjab and 70% of the total production is covered with the Kinnow (Niaz et al., 2004). Citrus industry of Pakistan is dominated by Kinnow mandarin (Khan et al., 2010). This is a prominent cultivar and contributes more than 70% of the citrus produce in the country (Ahmed et al., 2007). In Pakistan about 12.78 tons per hectare is the average yield of the citrus while the potential yield of the citrus is about 18-20 tons per hectare (PHDEB, 2006). In Pakistan, 187 varieties of citrus are commercially available and among all these citrus varieties kinnow is one of the most popular varieties. Pakistan is a 6th largest kinnow producer of the world with total plantation area of 8.6453 million hectares giving 123.69 million tons production during the year 2010-11 (FAO, 2010). In Punjab mostly used the root stock of rough lemon (Citrus jambhiri). It is a rich source of vitamins, minerals and carbohydrates, which are fundamentally requirement for human health. Citrus fruit has been reported to avoid lungs, liver, heart diseases, skin cancer, birth defects and also a

balanced healthy life routine (Ghirdharilal, 2000). Kinnow is mainly grown in the central Punjab covering an area of 95% in the district of Sargodha, Bhalwal, Sahiwal, Khanewal, Layyah, and Toba Tek Singh. Kinnow demands a shorter crop season (December-April) start of winter brings abundant availability of this fruit. Kinnow which is present in large quantities over a short harvesting period increase the risk of high post- harvest losses. The post-harvest losses are significantly higher and ranges from 35-40%. Citrus has a good demand internationally and foreign countries. Citrus has a high nutritional value and rich source of vitamins A and C along with sugar contents, organic acids and minerals like calcium and magnesium in proper amount (Niaz *et al.,* 2004). Pakistan has good agro-ecological environmental diversity, which is favorable for the production of almost thirty one kinds of fruits including dates, citrus mangoes, and apple and guava accounts for 73% of the total annual production of fruits.

In production system many changes have been compulsory to see the needs of growing markets and the demands of new challenges, like that change in organoleptic concerns, unexpected drought and cold stresses, outspread of pests and diseases and raises of political barriers. Subsequently, production limits have been overcome by the use of grafted plants to replace seedlings, changes in rootstocks, selection of new cultivars, and charges in diplomatic battles (Moreira, 1980).

The pathological factors involved in low yield of citrus include fungi, bacteria, nematodes and virus etc., which are causing different citrus disorder (Mordue, 1971). Among all these pathological factors fungi are the big cause of poor quantity and quality. Among the citrus diseases, citrus scab, melanose, anthracnose, greasy spot, citrus decline, citrus greening, citrus canker and blemish are the most common. The disease incidence from different countries has been reported to be 32% in South Africa, 64.6% in Costa Rica during 1990 (Arauz *et al*., 1994).

In wither-tip disease the branches and twigs looked to have been burned by fire. Drying of twigs from top to down-ward. Dry twigs are ash colored. While in citrus anthracnose affected fruit show tear stain symptoms (Agrios, 2005). *Colletotrichum gloeosporoides* produce water-soaked, round, brown colored and sunken lesions on the surface of the ripening fruits (Barker and D. Pitt, 1988).

Sporulation in *C. gloeosporoides* follows two ways, saprophytic and pathogenic (Barhoom and Sharon, 2004). Pathogenic germination occurs on plants characterized through mitosis followed by growth of single germ tube. This process started immediately and results in the development of appresoria. Saprophytic germination takes place in rich medium and it takes longer period of time with the development of two germ tubes that arise from opposite sides of the spore. But these germ tubes do not form appresoria ultimately plants are not infected.

Initially *C. gloeosporoides* was thought to be responsible for post-harvest anthracnose, but later on it was also reported as pre-harvest pathogen having symptoms such as: stem end rot of fruit, wither-tip and tear stain on citrus fruits (Huang *et al*., 2013). *Colletotrichum spp.* responsible for anthracnose and post-harvest losses on various tropical, subtropical and temperate fruits (Freeman *et al*., 1996). In citrus *C. gloeosporoides* cause post-harvest anthracnose (Brown *et al*., 1977). It is considered as a general pathogen that is linked with inactive infections and post-harvest diseases on numerous fruits such as avocado, guava, papaya, mango, apple, passion fruit, grapes, cashew and citrus (Simmonds, 1965).

The industry of citrus is one of the major fruit industries all over the world, hence knowledge about pathogens associated with citrus crop is very essential (Wang *et al*, 2012). (Dean *et al*, 2012) reported that *colletotrichum* genus is eighth most important plant pathogenic fungal group. Worldwide it is most important fungal plant pathogenic genera economically (Sutton *et al*, 1992). (Weir *et al*, 2012) observed that *colletotrichum gloeosporoides* is very common pathogen all over the world, also associated with citrus spp. for key lime anthracnose and postharvest diseases.

Keeping in view above all the facts, the study will be planned with following objectives:

- Molecular diversity analysis of *colletotrichum spp.*
- Assessment of the potential for disease development of *colletotrichum spp.* in different species of citrus fruits.

Materials and methods

1 Collection of diseased samples

For the collection of diseased samples showing characteristics symptoms of wither-tip disease and on twigs and branches survey of citrus orchards was carried out at three different places UAF, Ayub agriculture research institute (AARI) and Khanuwana respectively. The infected plants were counted separately in each orchard. The disease incidence was recorded by following formula.

$$\text{Disease Incidence} = \frac{\text{No. of infected plants}}{\text{Total no. of Plants}} \times 100$$

(Cardoso *et al*., 2004)

2 Disease rating scale

To measure the potential for disease development of *colletotrichum spp.* in four different species of citrus fruit following diseases rating scale was used.

$$\text{Percent Disease Index} = \frac{\text{Sum of Numerical Rating}}{\text{TNo. of Fruits Examined} \times \text{Maximum Grade}} \times 100$$

(Mayee and Datar, 1986)

Table 1: Disease rating scale for disease assessment

Grade	Fruit Area Effected
0	No Infection
1	Up to 5%
2	6-10%
3	11-20%
4	21-50%
5	More than 50%

3 Isolation and purification of pathogen

First of the infected samples were bought into the laboratory of department of Plant Pathology, UAF. After that the infected parts of twigs were cut in to small pieces measuring about 0.5 cm and then surface sterilization was done with 0.1 percent sodium hypochlorite solution for the removal of all kinds of saprophytic pathogens. Then these samples were rinsed twice for two minutes in distilled water. After that samples were transferred to petri dishes containing autoclaved potato dextrose agar (PDA) and incubated at 30±1°C. After 5-7 days colony growth of *C. gloeosporioides* will appear on petri dishes. After 15 days mature colony growth was formed on petri dishes having fruiting bodies of *C. gloeosporoides*. Then mycelium of pathogen will be transferred to slants containing PDA for purification and further use. The pure culture of *C. gloeosporioides* was maintained in the refrigerator and sub-cultured periodically during the course of this study.

4 Morphological identification

For morphological identification and spore confirmation of *C.gloeosporoides* microscopy was carried under microscope. Fruiting bodies were formed on petri dishes after 10-15 days.

5 Inoculum Multiplication

Culture of *C. gloeosporioides* from one glass cavity blocks were cut using sterilized transfer needle approximately into 4 cm strips and placed into a disinfected 250 ml flask having 100 ml distilled water. The flask was then shaken vigorously for 5 minutes and left to settle for 10 min. One and half ml of liquid from inoculated flask was poured onto a petri plates containing fresh autoclaved PDA. Then the glass cavity blocks were shivered slowly so that inoculums complete by covers the surface of PDA. The inoculated glass cavity blocks were placed in an incubator at 30±1°C. Almost a week after there was abundant sporulation was come over the entire of PDA.

6 Preparation of Spore Suspension

After spore confirmation of *C. gloeosporoides* under microscope, spore suspension has to be prepared to carry pathogenicity test on citrus fruits. For the preparation of spore suspension mature culture of pathogen 10-15 days aged was taken. Pour sterilized distilled water, on petri dish having mature culture of pathogen which was cooled after sterilization. After some time spores were automatically come on the surface of petri dish. Now spore suspension was collected in falcon tube of 45 ml. Now this spore suspension was ready for inoculation on citrus fruits.

7 Pathogenicity test

Pathogenicity test was carried out on healthy fruits of four different varieties of citrus (Kinnow, Musami, Red Blood and Shakri). Healthy fruits were collected from market and brought in the laboratory, washed thoroughly with water and scrubbed by hand to remove as many naturally formed appresoria as possible. Surface sterilization of these fruits was done with 0.1 percent sodium hypochlorite solution for the removal of all kinds of saprophytic pathogens. After that these fruits rinsed with sterilized distilled water and dried. Then these fruits were inoculated with spore suspension of *C. gloeosporoides* isolate. For inoculation of spore suspension on fruits, first of all these fruits were injured through pin-prick method. Then spore suspension was sprayed on fruits through hand sprayer. After inoculation, fruits were incubated for 72 hours at 25°C under humid environmental conditions in a humid chamber. For maintenance of humidity in a chamber fruits were covered with polythene sheet. Inoculated sites on all fruit were examined after 24, 36, 48 and 72 hours for the presence of anthracnose lesions. Fruits were rated as positive for anthracnose when sunken, necrotic lesions about the size of water droplets produced.

Fruits were rated as negative, did not differ from the non-inoculated controls. Re-isolation was made from each inoculated site using the selective medium for *Colletotrichum spp.* thus obtained cultures was compared with original cultures and observed morphologically under microscope to confirm the pathogen.

8. DNA extraction

To check out the molecular diversity among different isolates of *C. gloeosporoides* first of all DNA extraction of fungal mycelia was done by following modified CTAB method.

9 Agarose gel electrophoresis

DNA samples were analyzed by electrophoresis in 1.0% (w/v) agarose gels (0.5X TBE buffer) detected by staining with ethidium bromide (100 μg/ml) and viewed under ultraviolet trans-illuminator and photographed using the Gel-Doc EZ imager.

10 Restriction fragment length polymorphism (RFLP)

After confirmation of isolated DNA samples Restriction fragment length polymorphism (RFLP) was carried out.

11 Statistical analysis:

Data was analyzed by analysis of variance (ANOVA) and the significance of differences within treatments were be separated by using LSD test at probability levels P= 0.05 (Steel *et al*., 1997). In field data was analyzed by analysis of variance (ANOVA) and the significance of differences within treatments were separated by using LSD test at probability levels P= 0.05 (Steel and Torrie, 1980).

Results and discussions

4.1 Sample Collection

Fig. 4.1 Infected tree showing typical symptoms of citrus wither-tip and Fig.4.2 Infected fruit showing typical symptoms of citrus anthracnose disease

For sample collection survey was carried out from three different places of district Faisalabad (UAF, AARI and Khnuwana). Samples were showing characteristics symptoms of wither-tip disease, drying of twigs and branches that start from top to downward, infected twigs showing silvery appearance and black lesions on them as shown in figure (4.1). While infected fruits show tear stain and water soaked like irregular spots on them as shown in figure (4.2).

4.2 Disease Incidence

The results that were obtained from survey of showed that maximum disease incidence was observed at Khanuwan (75%), while minimum diseases incidence was observed at UAF (50%) as shown in table (4.1).

Table 4.1: Disease Incidence

Locality	No. of Plants observed	Infected Plants	Disease Incidence
UAF	20	10	50%
AARI	25	15	60%
Khanuwana	40	30	75%

4.3 Isolation and identification of Pathogen

The infected samples were surface sterilized and then rinsed twice in distilled water after that incubated on media plates at 30±1°C. After 7 days colony growth was observed on pathogen. Initially colony growth was observed whitish to light gray in color, as the culture become aged color of colony changed from light gray to dark gray in color as shown in figure (4.3). After 15 days spores were formed in culture of *C. gloeosporoides* and identification was done through microscopy by slide formation as shown in figure (4.4).

4.4 *In vitro* evaluation of four different citrus varieties against *C. gloeosporoides*

In vitro evaluation of four different varieties (Kinnow, Musami, Red Blood and Shakri) was carried out on fruits of theses varieties under CRD to check out the response against *C. gloeosporoides.* Healthy fruits of citrus varieties were collected from market; surface sterilization was done and inoculated with different isolates of *C. gloeosporoides* by casing injury

on fruits with pin-prick method under *in vitro* conditions. Percent diseases index was measured according to disease rating scale.

4.4.1 ANOVA for *in vitro* evaluation of Red Blood against *C. gloeosporoides*

The ANOVA table (4.2) shows that, fruit and days showed significant response in the development of *C. gloeosporoides.* The *in vitro* experiment was done under CRD with 3 replications. All the treatments were statistically at par.

Fig. 4. 3: Pure culture of *C. gloeosporoides* showing typical colony growth of *Colletotrichum spp.*

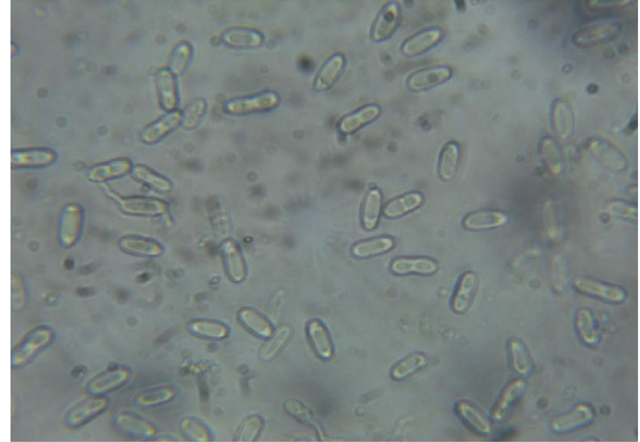

Fig. 4.4 Spores of *C. gloeosporoides* with obtuse ends and hyaline in nature

4.4.2 PDI of *C. gloeodsporioides* on Red Blood

The table (4.3) shows the percent disease index (PDI) of *C. gloeosporoides* on Red Blood after duration of 1, 2, 3 and 4 days. The maximum PDI (4.00) was recorded at day 4 while minimum PDI (0.71) was recorded at day first.

Table 4.2: ANOVA for the response of Red Blood against *C. gloeodsporioides*

Source of Variation	Degree of Freedom	Sum of Squares	Mean Sum of Squares	F-Value	P-Value
Fruit	6	8.35	1.39	18.47	0.00**
Day	3	42.39	14.13	187.42	0.00**
Error	18	1.35	0.07		
Total	27	52.10			

Table 4.3: Percent Disease Index of *C. gloeodsporioides* on Red Blood

DAYS	PDI
1	0.71d
2	1.71c
3	2.86b
4	4.00a

Alpha 0.05
Standard Error for Comparison: 0.1468
Critical T Value: 2.101
Critical Value for Comparison: 0.3084

4.4.3 ANOVA for *in vitro* evaluation of Musami against *C. gloeosporioides*

The ANOVA table (4.4) shows that, fruit and days showed significant response in the development of *C. gloeodsporioides.* The *in vitro* experiment was done under CRD with 3 replications. All the treatments were statistically at par.

Table 4.4: ANOVA for the response of Musami against *C. gloeosporioides*

Source of Variation	Degree of Freedom	Sum of Squares	Mean Sum of Squares	F-Value	P-Value
Fruit	6	3.71	0.61	2.05	0.00**
Day	3	49.82	16.60	55.07	0.00**
Error	18	5.42	0.30		
Total	27	58.96			

4.4.4 PDI of *C. gloeodsporioides* on Musami

Table 4.5 Percent Disease Index of *C. gloeodsporioides* on Musami

DAYS	PDI
1	0.85d
2	1.71c
3	3.28b
4	4.29a

Alpha 0.05
Standard Error for Comparison 0.2935
Critical T Value 2.101
Critical Value for Comparison 0.6167.

The table (4.5) shows the percent disease index (PDI) of *C. gloeosporoides* on Musami after duration of 1, 2, 3 and 4 days. The maximum PDI (4.29) was recorded at day 4 while minimum PDI (0.85) was recorded at day first.

4.4.5 ANOVA for *in vitro* Kinnow of against *C. gloeosporioides*

The ANOVA table (4.6) shows that, fruit and days showed significant response in the development of *C. gloeodsporioide*. The in vitro experiment was done under CRD with 3 replications. All the treatments were statistically at par.

Table 4.6: ANOVA for the response of Kinnow against *C. gloeodsporioides*

Source of Variation	Degree of Freedom	Sum of Squares	Mean Sum of Squares	F-Value	P-Value
Fruit	6	7.21	1.20	3.84	0.00**
Day	3	8.85	2.95	9.42	0.00**
Error	18	5.64	0.31		
Total	27	21.71			

4.4.6 PDI of *C. gloeodsporioides* on Kinnow

The table (4.7) shows the percent disease index (PDI) of *C. gloeosporoides* on Kinnow after duration of 1, 2, 3 and 4 days. The maximum PDI (1.57) was recorded at day 4 while no PDI (0.00) was recorded at day first.

4.4.7 ANOVA for *in vitro* evaluation of Shakri against *C. gloeosporoides*

The ANOVA table (4.8) shows that, fruit and days showed significant response in the development of *C. gloeodsporioides.* The in-vitro experiment was done under CRD with 3 replications. All the treatments were statistically at par.

Table 4.7: Percent Disease Index of *C. gloeodsporioides* on Kinnow

DAYS	PDI
1	0.00c
2	0.57bc
3	0.71b
4	1.57a

Alpha 0.05
Standard Error for Comparison 0.2993
Critical T Value 2.101
Critical Value for Comparison 0.6288

Table 4.8: ANOVA for the response of Shakri against *C. gloeodsporioides*

Source of Variation	Degree of Freedom	Sum of Squares	Mean Sum of Squares	F-Value	P-Value
Fruit	6	1.92	0.32	2.45	0.00**
Day	3	40.39	13.46	102.82	0.00**
Error	18	2.35	0.13		
Total	27	44.67			

4.4.8 PDI of *C. gloeosporoides* on Shakri

The table (4.9) shows the percent disease index (PDI) of *C. gloeosporoides* on Shakri after duration of 1, 2, 3 and 4 days. The maximum PDI (4.14) was recorded at day 4 while no PDI (1.00) was recorded at day first.

4.4.9 PDI of *C. gloeosporoides* on all four varieties of citrus

The table (4.10) shows the percent disease index (PDI) of *C. gloeosporoides* on all four varieties of citrus after the duration of 1, 2, 3 and 4 days. This table shows that after day 1 maximum PDI was showed by Shakri (1.00) while least PDI was showed by Kinnow (0.00). While after 4 days maximum PDI was showed by Musami (4.29) and minimum PDI showed by Kinnow (1.57) against the tested fungus (*C. gloeosporoides*).

Table 4.9: Percent Disease Index of *C. gloeodsporioides* on Shakri

DAYS	PDI
1	1.00d
2	2.00c
3	3.28b
4	4.14a

Alpha 0.05
Standard Error for Comparison 0.1934
Critical T Value 2.101
Critical Value for Comparison 0.4064

Table 4.10: Response of all four citrus varieties against *C. gloeosporoides*.

Days	Red Blood	Musmai	Kinnow	Shakri
1	0.71d	0.85d	0.00c	1.00d
2	1.71c	1.71c	0.57bc	2.00c
3	2.86b	3.28b	0.71b	3.28b
4	4.00a	4.29a	1.57a	4.14a
LSD	0.3084	0.6167	0.6288	0.4064

4.5 Genomic DNA Isolation

To check molecular diversity among different isolates of *C. gloeosporoides,* Genomic DNA extraction from fungal mycelia was done by CTAB method. DNA extraction of seven different isolates of *C. gloeosporoides* was carried out and confirmed by gel electrophoresis.

4.6 Restriction Fragment Length Polymorphism (RFLP)

To check genetic diversity of theses isolates Restriction Fragment Length Polymorphism (RFLP) was performed by using restriction enzyme *EcoR* I and *Pst* I. The RFLP results show that single type of *Colletrotricum spp.* is prevailing in district Faisalabad.

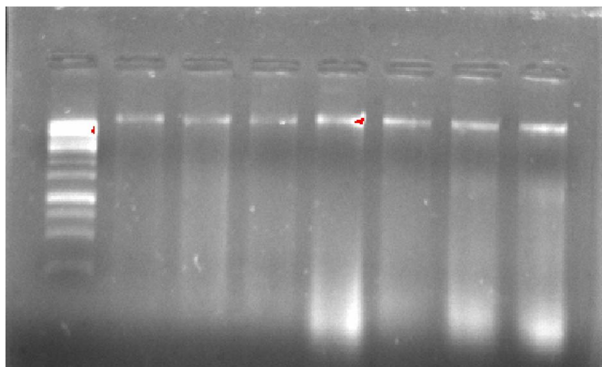

Fig. 4.5 Genomic DNA of Seven Isolates of district Faisalabad

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