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IDENTIFICATION AND FREQUENCY OF FUNGI ASSOCIATED WITH CITRUS DECLINE IN PUNJAB, PAKISTAN

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ABSTRACT

Background Citrus is an important fruit crop of Punjab province in Pakistan as more than 90% citrus is grown in this region. Citrus decline, a syndrome, is currently prevalent in all areas of Punjab and is caused by several biotic and abiotic factors, and fungus as pathogen may also be associated with it. The objective of current research was the identification of fungi associated with citrus decline and their frequency with declined trees.

Methodology Survey of different citrus growing areas of Punjab province was conducted for the collection of disease specimens. The collected samples were used for the isolation of the fungi using standard methods. Most of the fungi were isolated on PDA (potato dextrose agar) media and *Phytophthora* spp. were isolated using PARP (pimaricin, ampicillin, rifamicin and pentachloronitrobenzene) media.

Results Twelve fungi, viz., *Colletotrichum gloeosporioides*, *Fusarium solani*, *Phytophthora nicotianae*, *Fusarium oxysporum*, *Phytophthora citrophthora*, *Lasiodiplodia theobromae*, *Diaporthe citri*, *Phoma tracheiphila*, *Fusarium smitectum*,*Geotrichum candidum*, *Cladosporium cladosporioides* and *Alternaria alternata* were isolated and identified from declined orchards. Percent frequency of fungus *C. gloeosporioides* was significantly ($P \leq 0.05$) higher (41.20%) in declined orchards followed by *F. solani* (36.57%). The least isolated fungus was *A. alternata* having 17.13% frequency.

Conclusions The study revealed that *C. gloeosporioides* and *F. solani* were found to be the most frequent fungi associated with declined citrus trees in Punjab province of Pakistan.

INTRODUCTION

Pakistan ranks 12th in citrus with more than 2.5 million tons production and is grown on an area of 510431 acres. Citrus is cultivated in all provinces of Pakistan, but most of the citrus is cultivated in province Punjab due to adequate irrigation water, favourable climatic conditions and higher population. Province Punjab is producing 2.31 million tons annually which is 98% of the total produce of the country (Memon 2017). Citrus fruit export is important source of foreign exchange for Pakistan and citrus export value of the country is 200 million US dollar per annum (Malik and Khan 2014).

Citrus yield per hectare in Pakistan is 10-12 tons while in the other citrus producing countries 26 tons per hectare has been reported (Iqbal and Kamal 2014). Citrus diseases that are caused by fungi, bacteria, viruses, nematode and spiroplasma might be the main reason for lower citrus yield. Among them, pathogenic fungi causing citrus diseases are most important. Several fungi are known to cause citrus diseases, worldwide. *Fusarium* dry rot is important soil born fungal disease caused by *F. solani*, and this disease affects the root system of plant due to which nutrient and water uptake ability of plant reduces. This fungus also produces toxins which move into the xylem system of the plant and cause vessel plugging. Other fungal pathogens are involved in soil borne diseases which are *D. metalensis*, *Armillariamella*, *Pythium* spp. and *Thielaviopsis* and may have negative effect on yield. Mal secco is vascular disease caused by fungi *P. tracheiphila*, first time it was observed in Greece in

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1880s. Systemically, it enters into the stomata and occupies the xylem, as a result wilting starts. Other symptoms of this disease including discoloration of wood, venial chlorosis, wilting of leaf, and twigs di-back . Brown spot of citrus, stem end rot of citrus and black rot of citrus are well known diseases of citrus which produce brown to black spots on fruit and leaves, in case of black rot, fruit is infected internally, these diseases are caused by *A. citri* (Mojerlou and Safaie 2012). Several species of *Colletotricum* are known to cause important diseases of citrus, most important disease caused by this pathogen is citrus anthracnose, characteristic symptom of this disease is withering of twigs and lesions on fruits and leaves (Guarnaccia et al. 2017). *Citrus melanose* is important fruit and foliar disease of citrus caused by *D. citri*, tear drop, blemishes and mudcake are the symptoms produced by this disease. This disease does not affect pulp of the fruit but it reduces the market value (Gopal et al. 2014). Citrus scab is caused by *Elsinoe fawcettii* and *E. faustralis* that affect citrus fruit, leaves and shoot badly under warm and humid weather (Gopal et al. 2014). *Phytophthora* is an important genus that is pathogenic to citrus tree. There are several species of *Phytophthora* that are responsible for citrus diseases but most important are *P. citrophthora* and *P. nicotianae* that are responsible for damping of young seedling as well as yield loss and slow decline of adult trees (Erwin and Riberio 1998). Gummosis is present in all citrus producing regions of the world and causing 10-30% losses every year (Munde et al. 2012).

The present study was planned for isolation, identification of different pathogenic fungi prevailing in different citrus growing areas of Punjab, as well as to determine the relative frequency of pathogenic fungi in different citrus growing areas of Punjab.

MATERIALS AND METHODS

Sample collection

A total number of 90 samples were collected from the healthy, partially declined and declined plants to confirm the presence of the fungi. For association of fungi with declined trees, six trees were sampled from each orchard and samples were collected from shoots, bark, leaves, twigs and rhizosphere of 10-35 years old trees. The samples were placed in polythene bags and labelled with required information such as farmer name, date, tree condition and area of orchard. Then samples were brought to laboratory of Fungal Culture Bank, College of Agriculture, University of Sargodha, Sargodha, Pakistan, for further processing.

Isolation of fungi

The collected samples were used for the isolation of the fungi by using standard method. Most of the fungi were isolated on PDA (potato dextrose agar) media and *Phytophthora* spp. was isolated using PARP (pimaricin, ampicillin, rifampicin and penta chloronitro benzene) medium.

Preparation of PDA media

For preparation of PDA, 20 g potato starch, 20 g agar and 20 g glucose were dissolved thoroughly in one litre water in autoclavable bottles. The medium was then sterilized for 20-25 minutes in autoclave at the temperature of 121°C and 15 pascal pressure. Medium was then cooled and poured 15 ml quantity of this medium in sterilized petri dishes. Samples were then surface sterilized by giving them washings in 0.1% $HgCl_2$ followed by washing in sterilized distilled water and gently placed these samples on molten PDA in plates. Five pieces were placed on each plate and these plates after covering with wrapping tape were kept at 25°C for 3-4 days. Colonized fungi around these pieces were examined under compound microscope (Abid et al. 2014).

Preparation of PARP medium

Corn meal agar 17 g was mixed in 1000 ml of distilled water. Mixture was autoclaved for 20 minutes at 121°C and 15 pascal pressure. Medium was allowed to cool below 50°C. Antibiotics were prepared separately in two flasks. In one flask, 5 mL penta-chloronitrobenzene (PCNB), 0.25 g ampicillin, 0.4 ml pimaricin were added and dissolved in 10 ml distilled water. In another flask, 1 ml dimethyl sulfoxide (DMSO) and 0.01 g rifampicin were added to distilled water. Both these antibiotics were poured to initially prepared corn meal agar media and mixed thoroughly by shaking (Mohsin et al. 2017).

Isolation of pathogenic fungi and their frequency

Pathogenic mycoflora were isolated by using three different isolation techniques, and frequency of individual fungus isolated from samples was determined by using the formula (Safdar et al. 2010).

$$PFF = \frac{\text{Number of specimens colonized by pathogen}}{\text{Total number pf specimens used}} \times 100$$

PFF: Percent frequency of fungi

Soil dilution plate

Soil dilutions were prepared by taking 1 g of soil and added to 9 ml of distilled water. From 1st dilution, 1 ml was added to another tube containing 9 ml of distilled water, in this way four dilutions were prepared. From fourth dilution, few drops of water were poured in petri plates having PDA while for isolation of *Phytophthora* spp., few drops were poured on PARP media. Plates were incubated at 25ºC (Arshi and Nasreen 2016).

Soil plate method
In soil plate method, small amount of soil (0.005-0.015 g) was taken and spread in petri plates having PDA and PARP media. Plates were incubated at 25ºC (Arshi and Nasreen 2016).

Tissue planting method and purification
Tissue planting method was used for the isolation of pathogen from infected tissues. Infected plant parts were washed under tap water and converted into small segments of 3 mm. These segments were dipped into 70% ethanol for surface sterilization for 2-3 minutes and then washed with distilled water for two times. Five samples were placed in petri plates containing culture media. Plates were incubated at 25ºC in incubator and growth was observed after every 24 hours (Ghosh and Shamsi 2014).

Purification
When growth initiated on PDA and PARP media, mycelial bits were transferred to petri-plates having PDA and PARP media for purification and multiplication of mycoflora. Plates were incubated at 25 ºC and growth was observed after every 24 hours (Choi et al. 1999).

Identification
Fungi were identified on the basis of macroscopic and microscopic characters. For macroscopic characters, plates were observed visually as well as under stereoscopic microscope to observe colony colour and pattern. For microscopic characters, glass slides were prepared and observed under compound microscope (10, 40 and 100x). Spore shapes, hyphae growth and growth patterns were observed (Hasnaoui et al. 2017).

RESULS

Isolation of fungi
Twelve fungi viz., C. *gloeosporioides*, *P. nicotianae*, *F. oxysporum*, *P. citrophthora*, *L. theobromae*, *D. citri*, *P. tracheiphila*, *F. semitectum*, *G. candidum*, *C. cladosporioides* and *A. Alternate* were isolated from different parts of declined citrus trees and rhizosphere soil. Percent frequency of fungus *C. gloeosporioides* was significantly ($P \leq 0.05$) high (41.20%) in declined orchards followed by *F. solani* (36.57%), *P. citrophthora* (35.65%) and *F. oxysporum* (32.94%). The least isolated fungus was *A. alternata* having 17.13% frequency (Table 1).

Percent frequency of C. gloeosporioides
Percent frequency of fungus *C. gloeosporioides* was significantly ($P \leq 0.05$) different in different citrus growing areas of Punjab. Frequency of *C. Gloeosporioides* was higher in Kotmomin (55.56%) followed by Multan (48.15%), Bhalwal and Faisalabad/Toba (40.74%), Sillanwali (37.04%) and Sahiwal (37.03%). Lowest percent frequency of this fungus was found in Shahpur (33.33%) (Figure 1A).

Percent frequency of F. solani
Percent frequency of fungus *F. solani* was significantly ($P \leq 0.05$) different in different citrus growing areas of Punjab. Frequency of *F. solani* was higher in Multan (44.44%) followed by Kotmomin (40.74%), Bhalwal, Faisalabad/Toba, Sahiwal and Sillanwali (37%). Lowest PF of this fungus was found in Shahpur (26%) (Figure 1B)

Percent frequency of P. citrophthora
Percent frequency of *P. citrophthora* was higher in Kotmomin (48.15%) followed by Bhalwal, Sargodha and Faisalabad (40.74%), Sillanwali (33.33%), Shahpur and Sahiwal (29.63%). Percent frequency of this fungus was lowest in Multan (22.22%) (Figure 1C).

Percent frequency of F. oxysporum
Frequency of *F.* oxysporum was higher in Kotmomin (37.04%) followed by Bhalwal, Shahpur, Sahiwal and Faisalabad (33.33%), but it was least frequent in Sargodha, Sillanwali and Multan (29.63%) (Figure 1D).

Percent frequency of P. nicotianae
P. nicotianae was most prevalent in Kotmomin (46.15%) followed by Shahpur (40.74%), Sargodha (37.04%), Bhalwal (33.33%), Faisalabad (29.63%), Sahiwal and Sillanwali (25.93%), while lowest in Multan (14.81%) (Figure 1E).

Percent frequency of L. theobromae
L. theobromae was most frequent in Bhalwal with percent frequency of 48.15% followed by Kotmomin and Shahpur (37.04%), Sargodha and Multan (29.63%), Sahiwal (25.93%) and Sillanwali (22.22%). This fungus was least frequent in Faisalabad (18.52%) (Figure 1F)

Percent frequency of P. trichophila
P. trichophila frequency was higher in Kotmomin (37.04%) followed by Shahpur and Faisalabad (33.33%), Sargodha, Sillanwali and Sahiwal (29.63%), and it was lowest in Bhalwal and Multan (25.93%) and (4.16%), respectively (Figure 2A).

Percent frequency of F. semitectum
*F. semitectum*was found to be most prevalent in Bhalwal (37.04%) followed by Kotmomin and Multan (33.33%), Sargodha, Shahpur and Faisalabad (29.63%) and Sahiwal (25.93%). It was less frequent

Table 1 Fungi isolated from declined citrus orchards and their percent frequency in Punjab Province, Pakistan

Species of fungi	Source	Percent frequency of fungi in declined citrus orchards
Colletotrichum gloeosporioides	Twigs	41.20 ± 2.57a
Fusarium solani	Soil/root	36.57 ± 1.51a
Phytophthora citrophthora	roots/soil	35.65 ± 3.63b
Fusarium oxysporum	Soil/roots	32.41 ± 0.92c
Phytophthora nicotianae	Soil/roots/trunk	31.94 ± 2.97c
Lasiodiplodia theobromae	Stem	31.02 ± 3.36c
Phoma tracheiphila	Stem	30.56 ± 1.36c
Fusariumsemitectum	Soil	30.09 ± 1.63c
Diaporthe citri	Fruit	29.67 ± 2.83c
Geotricum candidum	Stem	28.74 ± 1.84c
Cladosporium cladosporioides	Twigs	25.67 ± 2.92d
Alternaria alternata	Leaves/Fruit	17.13 ±1.20e

Same letters in column are not statistically different according to Fisher's protected least significant difference (P = 0.05).

in Sillanwali (22.22%) (Figure 2B).

Percent frequency of D. citri

D. citri was most prevalent in Kotmomin (44.44%), Shahpur and Sahiwal (33.33%) followed by Sargodha (29.63%), Bhalwal and Sillanwali (25.93%), and Multan (22.22%). It was least frequent in Faisalabad (18.52%) (Figure 2C).

Percent frequency of G. candidum

*G. candidum*was higher in Kotmomin (37.04%) followed by Sillanwali (33.33%), Faisalabad (29.63%), Sargodha (29.63%), Shahpur and Sahiwal (25.93%). It was least frequent in Bhalwal and Multan (22.22%) (Figure 2D)

Percent frequency of C. cladosporioides

C. cladosporioides was most prevalent in Bhalwal (37.04%) followed by Kotmomin (33.33%), Sargodha (29.63%), Shahpur and Faisalabad (25.93%), Sahiwal (22.22%) and Sillanwali (18.52%), while this was least prevalent in Multan (11.11%) (Figure 2E).

Percent frequency of A. alternata

Frequency of *A. alternata* was significantly ($P \leq 0.05$) different in different citrus growing areas of Punjab (Figure 2E). *A.* alternata was significantly higher (22%) in Kotmomin followed by Sahiwal and Faisalabad/Toba Tek Singh (19%), Bhalwal and Sargodha (18%), Sillanwali (14%). The frequency of *A. alternata* was found very low (11%) in Shahpur (Figure 2F).

DISCUSSION

Different pathogenic and saprophytic fungi were found to be associated with declined trees. Most of the fungi found associated with declined trees during this study were in agreement with previous findings (Safdar et al. 2010). These fungi were reported to be the cause of decline syndrome in mango too (Ploetz et al. 1996). The results of present study indicated that *C. gloeosporioides* was the most frequent fungi in all citrus growing areas of Punjab. *C. gloeosporioides* is a weak parasite but it becomes pathogenic on plants when they become weak due to nutritional deficiency (Hu et al. 2015). This fungus has been reported to be the cause of citrus decline in recent study in Pakistan (Fayyaz et al. 2018). Black coloured pustules on twigs and branches, and chlorosis are the common symptoms produced by this fungus on citrus tree (Fayyaz et al. 2018). Du et al. (2017) reported that *C. gloeosporioides* could cause 25-35% yield losses in citrus. It has wide host rage including citrus, mango, guava, passion fruit and coffee (Sharma and Kulshrestha 2015). Our results also indicated the presence of two species of genus Phytophthora in citrus growing areas of Punjab, Pakistan. These species were identified as *P. citrophthora* and *P. nicotianae*. These *Phytophthora* species are important fungal pathogens causing citrus gummosis and have been reported to be the vital cause of citrus decline (Mounde et al. 2012). *Phytophthora* spp. are causing heavy yield losses all around the world. These fungi attack the fibrous roots of the plant and could cause 30% yield losses in citrus crop (Graham et al. 2013). It has also been reported that *Phytophthora* spp. caused collar and root rot in woody plants (Jung et al. 2005). *Phytophthora* spp. in combination with citrus greening disease could cause severe citrus decline (Graham et al. 2013). It has also been evident from different researches that citrus decline severity also increases by the attack of *Phytophthora* spp. when there is insect-pest attack on citrus rootstock (Graham et al. 2003). *Phytophthora* spp. are water borne fungal pathogens and are very devastating due to their nature

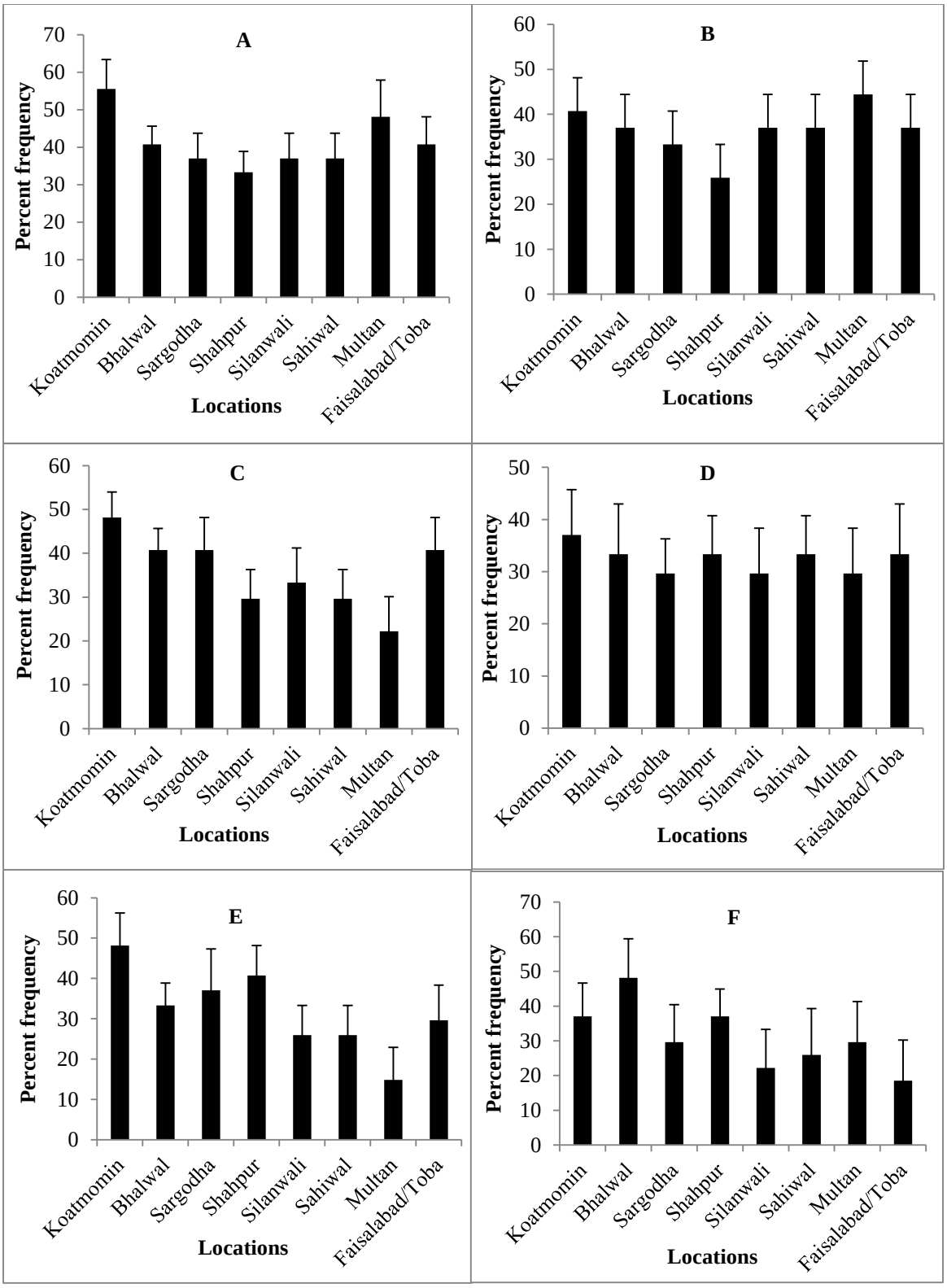

Figure 1 Frequency of *Colletotrichum gloeosporioides* in different citrus growing areas of Punjab (1A) *Fusarium solani* (1B) Phytophthora *citrophthora* (1C**)** *Fusarium oxysporum* (1D) *Phytophthora nicotianae* (1E) *Lasiodiplodia theobromae* (1F)

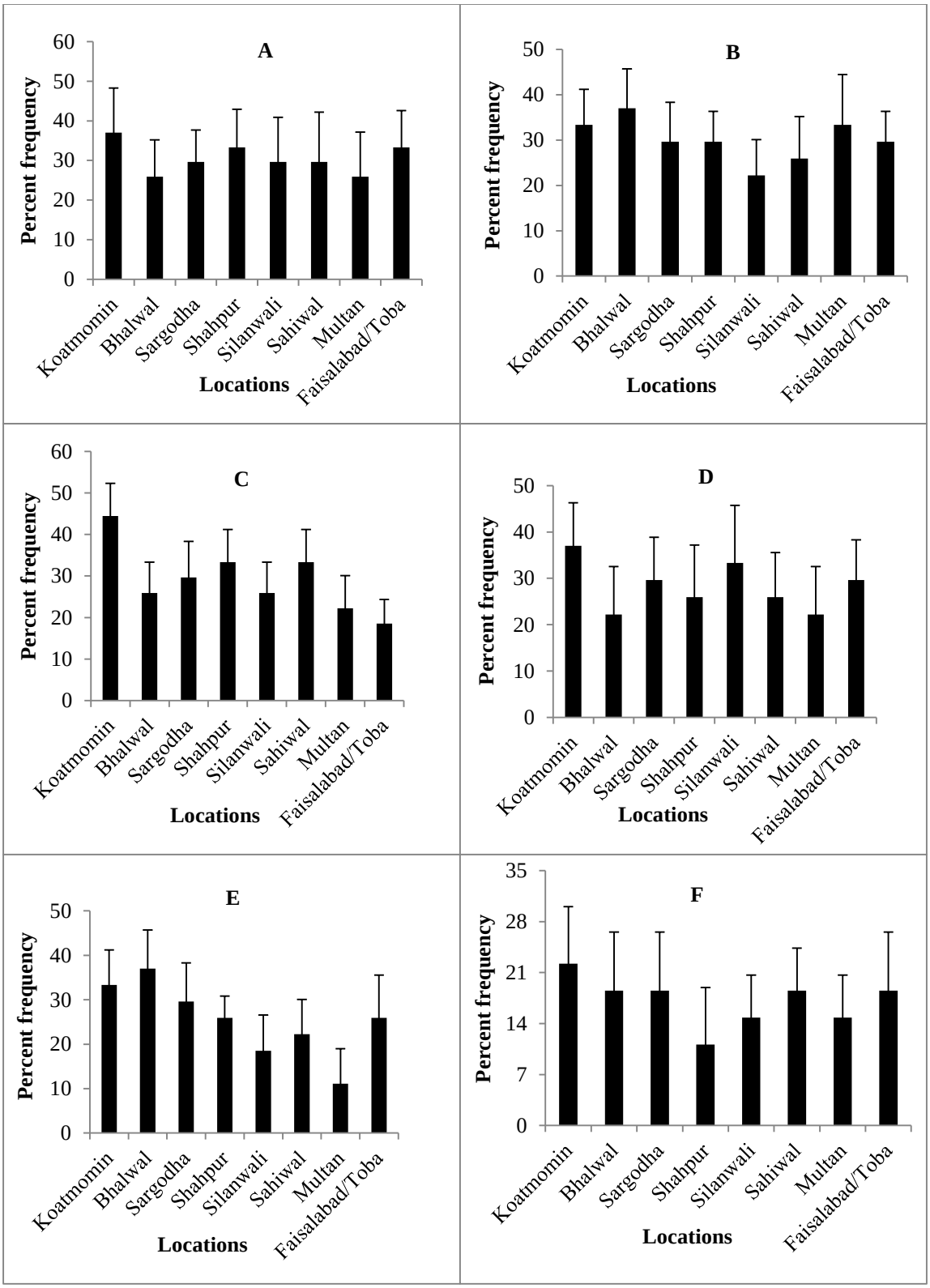

Figure 2 Frequency of *Phoma tricophila* in different citrus growing areas of Punjab (2A) *Fusarium semitectum* (2B) *Diaporthe citri (2C) Geotricum candidum* (2D) *Cladosporium cladosporioides* **(**2E**)** *Alternaria citri* (2F)

of rapid production of inoculum (motile zoospores, chlamydospores and oospores for their survival outside plant tissues); wide host range; availability of host plant all around the year; ability of single species to cause diseases in multiple hosts; and ability to cause more than one disease in single host (McConnell and Balci 2014).

From genus *Fusarium*, three important species viz., *F. oxysporum*, *F. Semitectum* and *F. solani* were identified. These are soil borne pathogens and have wide host range (Edel-Hermann and Lecomte 2019). These species are known to cause dry rot, root rot and wilt in several host plants, invade host plant through root or stem, and spread quickly in vascular system resulting in plant wilting. Infected roots become discoloured, water soaked, soft, and cortex is shed off (Abdel-Monaim et al. 2014; Sun et al. 2017). El–Mohamedy et al. (2012) reported that in Egypt *F. solani* was among serious pathogens of citrus. It was affecting 11.8% mandarin tress and causing 39.6% yield losses. Citrus tree death has been reported by *Fusarium* spp. in Tunisia (Hannachi et al. 2014). It is possible that these species could be the main cause of citrus decline here in Pakistan.

A. alternata was another fungus isolated from citrus decline trees during the study. This fungus has been reported to cause brown spot on citrus tree (Peever et al. 2005). Distinct lineages of this fungus also cause black rot of citrus (Peever et al. 2005). This fungus also caused fruit drop in pomegranate trees by weakening them (Ezra et al. 2018). This fungus produced complex symptoms on citrus trees and succumb them to death (Timmer et al. 2003). Fungi viz., *L. theobromae*, *P. tracheiphila*, *D. citri*, *G. candidum* and *C. cladosporioides* were also identified. There are several reports that these fungi were allied with several tree declines (Hay et al. 2015). These plant pathogenic fungi caused different structural changes in root system of the plant resulting in declining symptoms. Further these fungi attacked the fibrous root system of the plant and induced heavy yield losses in citrus. Our study demonstrated that citrus decline negatively impacted yield of citrus. This decline in citrus yield may be attributed due to loss of fibrous roots of declined trees (Graham et al. 2013).

CONCLUSIONS

The current study revealed that different fungi were associated with citrus decline but *C. gloeosporioides* and *F. solani* were found to be most frequent.

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