

SURVEY AND INVESTIGATION OF DIFFERENT CITRUS GROWING AREAS FOR CITRUS SUDDEN DEATH SYNDROME

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ABSTRACT

A survey was carried out in Bhalwal, Kot Momin, Sillanwali, Shahpur, Sargodha and Faisalabad. Disease incidence was found to be the maximum at Kot Momin (3%) followed by Bhalwal (2.41%) and Sargodha (2%) however it was low at Silanwali, Faisalabad and Shahpur viz. 1.92, 1.6 and 1.48% respectively. Colonization percentage of the *Fusarium semitectum* in 1536 tissues of 285 samples was counted to be 36.84%, *Fusarium solani* (15.10%) was the second frequent fungus. *Helminthosporium* spp. (5.20%) and *Phytophthora* spp., were the third and fourth frequent fungi respectively. These all fungi were isolated frequently from roots and collar region. *Aspergillus flavus* and *Aspergillus niger* were also isolated from the samples but mostly from twigs. *Rhizopus* spp. was the least frequent fungi. The maximum nematode (*T. semipenetrans*) population was recorded in Bhalwal (65.38) followed by Kot Momin (61.53). In some orchards nematode population was above economic threshold level. The highest number of females (943) of *T. semipenetrans* and number of J₂ (12624) were found from Bhalwal. The nematode infestation recorded in Silanwali, Shahpur, Sargodha and Faisalabad was calculated as 60%, 52.26%, 54% and 62% respectively.

Key words: Survey, citrus growing areas, citrus sudden death syndrome

INTRODUCTION

Citrus fruit belonging to the family *Rutaceae*, is grown in tropical and subtropical climate all over the world. Origin of citrus is believed to be southern slope of Himalayan region, the entire north eastern region of India and adjacent China (Gmitter *et al.*, 1990). It stands first in area and production among the tree fruits in world. It is essential for human health due to its high nutritional value as a rich source of vitamin C, sugar, organic acids, amino acids and minerals like calcium and magnesium. It is being used as fresh and in processed form worldwide. China, United States, Mexico and Spain were the biggest citrus producers in the 2007- 08. (<http://users.kymp.net/citruspages/introduction.html>). In Pakistan it is the largest group of fruits grown over an area of 199.4 thousand hectares with an annual production of 2294.5 thousand tonnes (Anonymous, 2008). Average yield of citrus in Pakistan is about 12.78 tones per hectare while the potential yield of citrus is 18-20 tones per hectare (Anon., 2006), hence there is a big gap between its average and potential yield. This yield gap is attributed to a number of cultural and environmental factors as well as infectious diseases, caused by different pathogens. Citrus is known to suffer from a number of diseases caused by diverse range of pathogens like fungi,

bacteria, virus and virus-like pathogens. Citrus sudden death (CSD) was first observed in 1999 in Minas Gerais and northern Sao Paulo States, Brazil (Fernandes and Bassanezi, 2001). This disease has been observed on sweet oranges (*Citrus sinensis* (L.) Osb.) and some mandarins (*C. reticulata* Blanco) (Fernandes and Bassanezi, 2001; Roman *et al.*, 2004). CSD is a serious emerging threat to citrus industry in Pakistan. It was therefore decided to conduct a survey of different important areas of Faisalabad and Sargodha districts to assess the CSD incidence.

CSD is most discussed all over the world. Its canopy symptoms are not specific, but have some typical characteristics like wilting and yellowing of leaves followed by defoliation and fruit drop. Dieback of branches starts from tip to the downward and branches turn yellow to brown, a healthy tree with a good crop suddenly wilts, turns yellow and dies rapidly. This phenomenon is often observed in conjunction with conditions that causes stress in the trees. Trees become unthrifty over time. CSD symptoms also develop on roots characterized by root rotting with shredded bark. Root rotting occurs on tap roots and laterals roots. Plant height remains unaffected by CSD. Affected roots appear dirty as compared to healthy roots because of soil particles and infected feeder roots remain stunted (Spina,

2008; Bassanezi *et al.*, 2003). The declined citrus trees have fewer fibrous necrotic roots (Nemec *et al.*, 1980; Valle, 1987 and Vegas *et al.*, 1988). Disease pattern in the field is usually uneven. The disease can occur in tree at any stage of growth.

MATERIAL AND METHODS

The survey was conducted in various citrus fields of Faisalabad and Sargodha districts in order to ascertain the disease severity and to collect the diseased samples. It was carried out in the main citrus growing areas like Bhalwal, Kot Momin, Silanwali, Sargodha, Shahpur and Faisalabad in 2009-10. Three orchards were selected at random from each location. Total 18 orchards were surveyed.

Disease prevalence and incidence were measured by the following formulae respectively;

$$\text{Disease Prevalence (\%)} = \frac{\text{No of orchards infected}}{\text{Total no of orchards inspected}} \times 100$$

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

Collection of diseased samples

Fungal pathogens Isolation: Two hundred and eighty five samples were collected from the healthy and infected trees to ascertain the association of different pathogens. In order to confirm the association of pathogens with the disease six trees were sampled from each orchard including healthy and infected trees. The samples consisted of roots along with feeder roots, bark at collar region and twigs from 15-25 years old trees in the areas mentioned earlier. Four sub samples of each part of a tree were taken to make a uniform composite sample. The samples were placed in paper bags and labeled with required data such as date, place, name of farmer, area of orchard, tree condition etc. Then samples were brought to laboratory for further studies.

Nematode isolation: From same six trees sampled for fungal pathogens isolation, soil with feeder roots was taken at a depth of 4-6 inches under canopy of tree for nematode isolation. From each tree 4-5 sub samples were collected and mixed thoroughly and a composite sample of 500g was taken in the polythene bag which were properly labeled with data such as location, date of collection, age of tree and condition of the tree. Three samples from each tree were collected. Polythene bags sealed loosely to maintain humidity and aeration were brought to Laboratory, Department of Plant Pathology, University of

Agriculture, Faisalabad for further studies and were stored at 10°C in a refrigerator to keep them fresh until further processing.

Isolation of fungi : Isolation of fungi was done in Plant Disease Diagnostic Laboratory, Department of Plant Pathology, University of Agriculture, Faisalabad. The collected samples were used for the isolation of fungi by usual isolation procedure (Ricker and Ricker, 1936). The infected samples comprised roots, leaves and bark bearing prominent symptoms was cut into small pieces of 4-5 mm length. Each sample was cut into 8-10 small tissues. The cut pieces were surface sterilized with 1% solution of sodium hypochlorite for 1-2 minutes. The sterilized pieces were then rinsed twice with distilled water and transferred on to filter paper in petri plates for drying. Then sterilized pieces were plated in Petri plates containing potato dextrose agar (PDA) medium (five pieces of specimens in one petri plate), using sterilized forceps. For cutting and transferring these pieces scissors and needle were also sterilized by dipping in methylated spirit and flaming several times.

All Petri plates were incubated at 27°C±2°C for 3-4 days. The fungi which colonized these pieces was purified and identified after consulting the relevant literature (Ellis, 1971).

The frequency of individual fungus isolated from each part from the cultured pieces was calculated using the following formula:

$$\text{Relative density of fungi (\%)} = \frac{\text{No. of pieces colonized by a pathogen}}{\text{Total number of pieces}} \times 100$$

Hyphal tips of fungal colonies, growing out from plated material were transferred aseptically to PDA slants and incubated again for about a week at 27°C±2°C, during which fungal growth appeared on the medium. For the identification of the isolates from diseased pieces of citrus trees, temporary mounts were prepared, initially in distilled water and then in cotton blue Lactophenol.

Isolation of citrus nematode (*T. semipenetrans*):

The isolation of nematodes from the soil and the root samples was carried out by using Whitehead and Hemming tray method (Whitehead and Hemming, 1965) and Baermann funnel method (McKenry and Roberts, 1985) respectively. Number of citrus nematode larvae at 2nd stage juveniles (J₂) from soil and roots and number of females/gram of roots were recorded. The data was recorded on the basis of number of 2nd stage juveniles/100 cm³ of soil, number of 2nd stage juveniles/gram of roots, number of females per gram of roots and relative density of nematodes by using the following formula:

$$\text{Relative density of nematode (\%)} = \frac{\text{No. of samples with } T. \textit{semipenetrans}}{\text{Total number of samples}} \times 100$$

RESULTS

A survey of different locations of two important citrus growing districts i.e. Faisalabad and Sargodha was conducted and three orchards were selected at random in each location, disease prevalence and incidence was calculated for the above said districts.

Disease Prevalence: The disease prevalence was calculated using the data recorded. The result showed that disease prevalence in Kot Momin, Bhalwal, Silanwai and Sargodha was 100% which revealed that all of the orchards visited were infected with CSD. Faisalabad and Shahpur showed 66.66% prevalence because two out of three orchards were infected whereas third orchard was healthy as shown in figure-1.

Disease Incidence: During the visit the total number

of trees from each orchard were counted and recorded. The total number of trees showing the decline symptoms, infected with CSD was recorded. Disease incidence was maximum at Kot Momin (3%) followed by Bhalwal (2.41%) and Sargodha (2%) as shown in figure-2. It was low at Silanwali, Faisalabad and Shahpur i.e. 1.92, 1.6 and 1.48% respectively as shown in figure-2.

Isolation, identification and purification of the pathogens

Isolation of the fungal pathogens: Colonization percentage of the *Fusarium semitectum* was found to be highest (36.84%) in 1536 tissues of 285 samples. *Fusarium solani* (15.10%) was the second frequent fungus whereas *Helminthosporium* spp. (5.20%) and *Phytophthora* spp., were the third and fourth frequent fungi respectively. These all fungi were in abundance isolated frequently from roots and collar region. *Aspergillus flavus* and *A. niger* were also isolated from the samples but mostly from twigs. *Rhizopus* spp. was the least frequent fungus as mentioned in Table.1.

Table.1: Colonization % of different fungi colonized on different plant parts of citrus trees

Sr. No	Name of Fungus	Name of Sample	Colonization Infection (%age)
1	<i>Fusarium semitectum</i>	Roots, collar region	36.84
2	<i>Fusarium solani</i>	Roots, collar region	15.10
3	<i>Helminthosporium</i> spp.	Roots, collar region	5.20
4	<i>Phytophthora</i> spp.	Roots, collar region	4.62
5	<i>Aspergillus flavus</i>	Twigs	4.16
6	<i>Aspergillus niger</i>	Twigs	3.71
7	<i>Rhizopus</i> spp.	Roots, collar region	0.65

The examination of infected parts of citrus trees significantly revealed the association of these fungi at different frequencies viz. *F. semitectum*, *F. solani*, *Phytophthora* spp., *Helminthosporium* spp., *A. flavus*, *A. niger* and *Rhizopus* spp. Majority of the samples yielded the *F. semitectum*, this was isolated from all locations. Keeping in view the highest isolation frequency of *F. semitectum* from all samples a comparison was also made between the different locations as shown in Table-2. Maximum colonization of *F. semitectum* was observed at Bhalwal followed by Sargodha, Silanwali, Kot Momin and Faisalabad respectively. However Shahpur showed the minimum colonization of *F. semitectum* as shown in fig-3.

Isolation of the Nematodes: The results of a survey of different locations indicated that maximum nematode population has been recorded from Bhalwal (65.38%) followed by Kot Momin (61.53%). In some of the orchards studied nematode population was above the economic threshold level. The nematode infestation recorded in Silanwali, Shahpur, Sargodha and Faisalabad was calculated as 60%, 52.26%, 54% and 62% respectively. This has been shown in the figure-4. The highest number of females of *T. semipenetrans* per gram of roots were found in Sargodha (991) followed by Bhalwal (943) as shown in the figure- 5. The number of J2 per gram of roots (12624) was found from Bhalwal shown in the figure-6. The Number of J2 of *T. semipenetrans* per

Figure.1: Disease prevalence in Sargodha and Faisalabad Districts

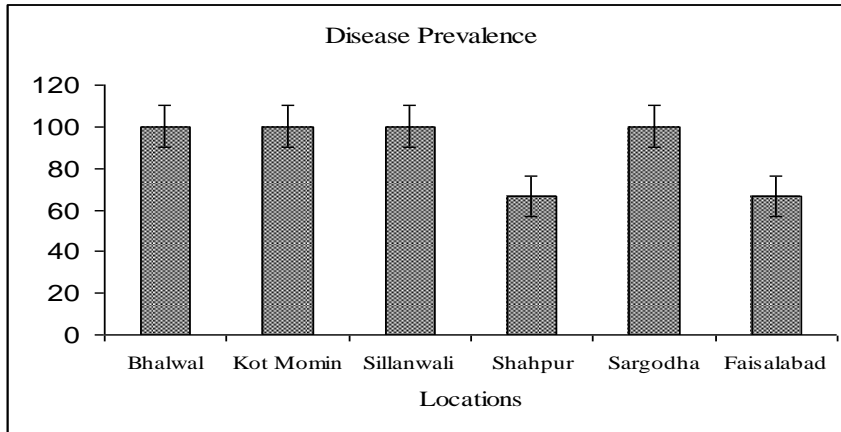


Figure.2: Disease incidence in Sargodha and Faisalabad Districts

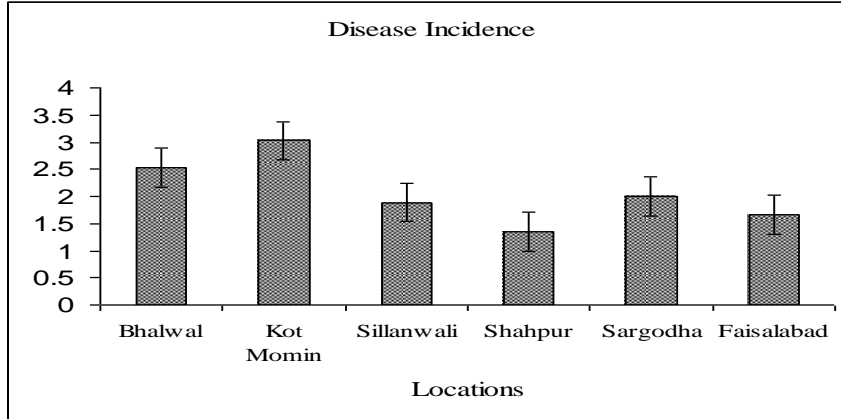


Figure.3: Frequency (%) of *F. semitectum* isolated from different plant parts citrus trees of different locations

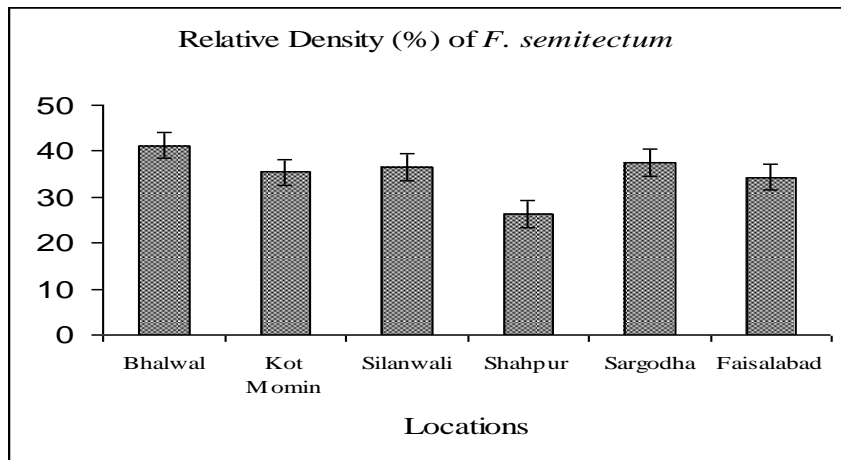


Figure.4: Relative density of citrus nematodes (*T. semipenetrans*) isolated from different locations

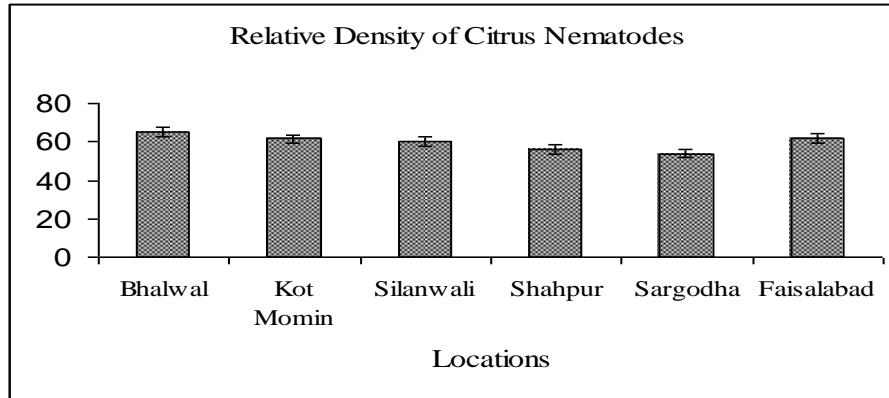


Figure.5: Number of females of Citrus nematodes (*T. semipenetrans*) per gram of roots isolated from different locations

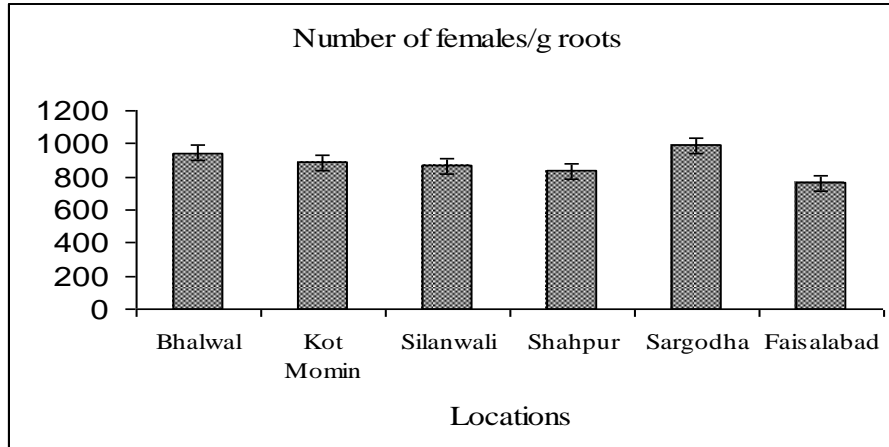


Figure.6: Number of Juveniles of Citrus nematodes (*T. semipenetrans*) per gram of roots isolated from different locations

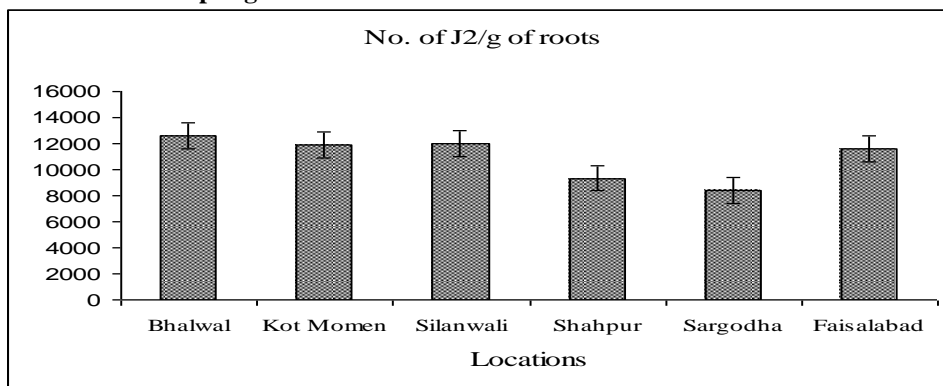
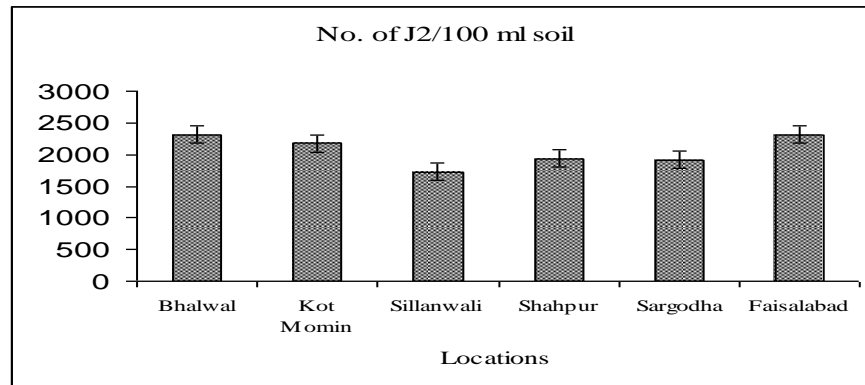


Figure.7: Number of Juveniles of Citrus nematodes (*T. semipenetrans*) per 100ml of soil isolated from different locations



100 ml of soil were recorded maximum at Faisalabad (2318) and Bhalwal (2315) as shown in figure-7.

DISCUSSION

Citrus Sudden Death is becoming a serious threat day by day in citrus growing areas of Pakistan. It is a complex disease and different pathogens have been reported in citrus rhizosphere. Considering this aggravated intensity of disease and hazard, a comprehensive survey was conducted on disease incidence in the two districts of Punjab which are mainly citrus growing areas in Pakistan. This disease had been reported in a few countries so there is the need of extensive work over its etiology and epidemiology, to avoid the heavy losses expected in future. Infected plants do not show prominent symptoms on early stage but when symptoms become prominent in just few days before death; usually treatment does not work. Disease prevalence and disease incidence was calculated in these districts. The variation may be due to the soil factors including soil texture and environmental conditions. Similar survey for slow decline was also carried out by Pervaiz *et al.*, 2003; Al-Azzeh *et al.*, 2005; Gene *et al.*, 2005 and they isolated the citrus nematodes and calculated the disease incidence. Our results regarding the nematode population are also in conformity with them.

The samples of roots, twigs and bark at collar region were taken from each tree. Colonization percentage of the *F. semitectum* in 1536 tissues of 285 samples was the maximum; other fungi isolated were *F. solani*, *Helminthosporium* spp., *Phytophthora* spp., *A. flavus*, *A. niger* and *Rhizopus* spp. isolated frequently from twigs.

The examination of infected parts of citrus trees revealed the association of these fungi at different frequency viz. *F. semitectum*, *F. solani*,

Phytophthora spp., *Helminthosporium* spp. *A. flavus*, *A. niger* and *Rhizopus* spp. From majority of the selected samples *F. semitectum* was the most dominant. Maximum colonization of *F. semitectum* was observed at Bhalwal followed by Sargodha, Silanwali, Kot Momin and Faisalabad. Shahpur showed the minimum colonization.

F. semitectum reported to be associated in mango malformation in Pakistan (Khaskheli *et al.*, 2008). Our results are also in conformity with Rensburg, 1996; Sherbakoff, 1953; Feder *et al.*, 1956; Labuscagne *et al.*, 1987). *F. semitectum* had been reported to be present in roots of different cultivated crops such as citrus (Nemec, 1987), peppers (Fletcher, 1994), beans (Silbernagel and Mills, 1990), peanut (Federico *et al.*, 2006), agricultural crops (Latiffah *et al.*, 2007), legumes (Embaby and Abdel-Galil, 2007) and bottle gourd in Pakistan (Sultana *et al.* 2009). The spread of most of *Fusarium* species depend upon the climatic factors (Backhouse *et al.*, 2002). *F. solani* has reported to be widely distributed in numerous native soils such as sub tropical, semi-arid and grassland soils (Burgess and Summerell, 1992; Zaccardelli *et al.*, 2008) and desert soil (El Gindy and Saad, 1990). *Fusarium* species found to be present in all the four soil types i.e. sandy loam, silt clay loam, silt loam and silt clay soils (Latiffah *et al.*, 2007).

Maximum nematode population was recorded in Bhalwal followed by Kot Momin. The highest population of females and number of juveniles of *T. semipenetrans* was recorded in Bhalwal. Nematode infestation was also recorded in Silanwali, Shahpur, Sargodha and Faisalabad. In Pakistan it was reported at Faisalabad in 1962 (Brown, 1962). Our results regarding the citrus nematode population in Punjab are in conformity with Pervaiz *et al.*, 2003; Iqbal *et al.*, 2006; Ahmad and Khan., 1999 and Chohan *et al.*, 2007.

Many nematode species have been reported to be parasitizing the citrus but *T. semipenetrans* was the most important on worldwide basis (Mani *et al.*, 1988; Al-Qasim and Abu-Ghabrich, 1995; Crozzoli *et al.* 1997; Verdejo and McKenry., 2004; Elekcioğlu, 2000; Gene *et al.*, 2005; Duncan, 2005; Al-Azzeh *et al.*, 2005). Nematode populations vary considerably each year (Bannon *et al.*, 1972). The citrus nematode, (*T. semipenetrans*) associated with citrus declined trees have been isolated from citrus rhizosphere all over the world including Pakistan (Gundy and Meagher, 1977; Heald and Bannon, 1987; Pervaiz *et al.*, 2003; Gene *et al.*, 2005). The present survey confirms the occurrence of citrus nematode in District Sargodha and Faisalabad and association of citrus nematode with CSD.

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