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Synergistic Effect of a Fungus, *Fusarium semitectum*, and a Nematode, *Tylenchulus semipenetrans*, on Citrus Decline

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Abstract.- Synergistic effect of a fungus *Fusarium semitectum* Berk & Rev. and a nematode *Tylenchulus semipenetrans* Cobb. was determined individually and concomitantly on citrus. Maximum reduction in the plant growth parameters as number of leaves (61.33%), root length (39.7%), shoot length (31.74%), fresh shoot weight (35.2%), fresh root weight (42.21%), stem diameter (47.78%) and number of feeder roots (50.09%) were observed in the plants having combined treatment of *F. semitectum* and *T. semipenetrans*. Nematode reproduction parameters as number of females/g roots (60.22%) and number of juveniles (J2)/g roots (59.97%) and number of J2/100 ml soil (61.34%) were maximum in the individual treatment of *T. semipenetrans*. Existence of synergistic relationship between *F. semitectum* and *T. semipenetrans* in citrus decline was confirmed.

Key words: *Fusarium semitectum*, *Tylenchulus semipenetrans*, synergistic effects, citrus decline.

INTRODUCTION

Citrus trees are known to be attacked by several soil borne plant pathogens either at seedling stage in nurseries or juveniles and mature trees in fields. Many nematode species have found to be parasitizing the citrus, but *Tylenchulus semipenetrans* Cobb. is the most important pathogen causing slow decline in citrus on a worldwide basis (Verdejo and McKenry, 2004; Duncan, 2005). Among the many problems limiting the citrus production, plant parasitic nematodes rank the highest (Abd-Elgawad *et al*., 2010). In Pakistan, citrus nematode was found to be widely distributed in all the citrus orchards of Faisalabad, Sargodha, T.T. Singh, and Sahiwal districts of Punjab. Infestation was above economic threshold level in Faisalabad and Sargodha districts (Pervaiz *et al.*, 2003; Iqbal *et al*., 2006).

Most of the workers have reported the pathogenicity of *Fusarium solani* (Mart) Apple and Wr. emend. Snyd. & Hans. to citrus roots from various parts of the world (Gundy *et al.*, 1963; Bannon, 1966; Nemec, 1978; Nemec *et al.*, 1980, 1986; Baker *et al.*, 1981). *F. solani* has been reported to attack all of citrus varieties (Conzulex *et al.*, 1997; El-Mohamedy, 1998; Catara and Polizzi, 1999). It has been reported to infect 11.6% of mandarin and caused 39.6% loss in fruit yield (El-Mohamedy, 1998).

Fusarium spp. are the most soilborne fungi that have commonly been associated with different varieties of citrus and can cause serious diseases (Abd-Elgawad *et al*., 2010). Some of these diseases are vascular wilt, feeder root-rot, dry root rot, root and stem rots, dieback and twig blight, all of cultivated citrus trees worldwide (Armstrong and Armstrong, 1975; Bender *et al.*, 1982; Labuschange and Koteze 1988; Nemec *et al*., 1989). Studies had shown that *Fusarium* spp. can be pathogenic on citrus roots alone (Nemec, 1975; Nemec *et al*. 1989; Morsy and El-Mohamedy, 2003) or in combination with nematodes (Martin, 1960; Feder and Feldmesser, 1961; Feldmesser *et al*., 1962; Van Gundy and Tsao, 1963; O'Bannon *et al*., 1967; Labuschange *et al*. 1989; Combrink *et al*., 1996; El-Mohamedy *et al*., 2005).

F. solani is associated with two types of syndromes namely dry root rot and chronic feeder root rot which cause slow decline of citrus (Bender *et al.*, 1982). Dry rot effect the crown and scaffold roots and chronic feeder root rot cause the gradual decline of tree canopy, wilting, defoliation, and die

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back of twigs. The fibrous roots turn soft and seems water soaked (Kore and Mane, 1992; Praksasm *et al.,* 1992; Verma *et al.,* 1999). Besides pathogenic to various crops, *Fusarium* pathogen produces mycotoxins that are harmful to humans and animals (Ueno 1987; Jenkins *et al*. 1988; Israel 1989; Nemec *et al.,* 1991; Achor *et al*., 1993; Wong *et al*. 1995).

The *Fusarium* spp. and *Phytophthora nicotiana* Breda de Haan (= *P. parasitica* Dastur) are commonly associated with root rot of citrus (Booth, 1971; Timmer &Menge, 1988). *F. solani* is universally present in the roots of citrus trees in the field (Labuschagne *et al.,* 1987). As *Fusarium* and *T. semipenetrans* both are soilborne pathogens so there must be the close association occurring between them. The role of *Fusarium* spp. and *T. semipenetrans* on root rot disease development on citrus plants were better defined in recent researches (Kore and Mane, 1992, El-Mohamedy, 1998; Catara and Polizzi, 1999; El-Nouby, 2002; El-Mohamedy *et al*., 2005). During a survey of Sargodha and Faisalabad districts maximum frequency (%) of the *Fusarium semitectum* (36.84%) along with a heavy population of nematode (*T. semipenetrans*) was recorded. In some orchards nematode population was above economic threshold level (Safdar *et al*., 2010).

Keeping in view the importance of synergistic relation between the pathogens, an experiment was designed to investigate the role of these two most frequently isolated pathogens in citrus decline.

MATERIALS AND METHODS

Preparation of inoculum of T. semipenetrans

Inoculum of *T. semipenetrans* was obtained from roots of declining citrus trees from citrus orchard in University of Agriculture Faisalabad and maintained on *Citrus jambhiri* Lush in green house. The inoculum consisted on suspension of live nematodes culture at J2 stage was obtained from soil and roots by Whitehead and Hemming tray method (Whitehead and Hemming, 1965) and Baermann funnel method (McKenry and Roberts, 1985).

Preparation of inoculum of F. semitectum

Isolates of *F. semitectum* obtained from declined rough lemon rootstocks (*C. jambhiri* Lush) of Faisalabad district were used in pathogenecity test. *Fusarium* inoculum consisting conidial suspension, was obtained by growing the cultures into potato-dextrose (PD) broth from 7-days old culture on potato-dextrose agar (PDA). The flasks containing PD broth were then inoculated with 14 day old *F. semitectum* culture using sterilized inoculating needle. Labeled flasks were placed in an incubator at 28±2^{o}C and observed daily for mycelial growth. After 12 days entire surface area of each flask was covered with mycelial growth of *F. semitectum.* After 12 days the inoculum of all the flasks was mixed and grinded in blender for 3-4 s to break mycelia into fragments. Then a suspension was obtained, containing spores (macroconidia) and fragments of mycelium. The spores were counted by haemocytometer, spore counting chamber.

Method of inoculation

Inoculation of T. semipenetrans

Inoculation with fresh culture of *T. semipenetrans* was done by soil application. Depending on the size of pots 4-6 holes up to middle of the pots near the root system of the plants were made with the help of pointed wood. The rootstocks were inoculated with 45 ml of water suspension containing freshly hatched 2nd stage juveniles of *T. semipenetrans* at the rate of 2000 juveniles per pot. The suspension was distributed equally in six holes in each pot and covered with soil after inoculation to prevent drying. The pots were irrigated daily with tap water carefully to prevent loss of nematodes through leaching or excessive drying.

Inoculation of F. semitectum

Inoculation with freshly prepared spore suspension of *F. semitectum* was done by soil application. Depending on the size of pots 4-6 holes up to middle of the pots near the root system of the plants were made with the help of pointed wood. The rootstocks were inoculated with 45 ml of water suspension containing spore suspension of *F. semitectum* at rate of 1.2×10^{7} conidia per ml per

pot. The suspension was distributed equally in each pot and covered with soil after inoculation to prevent drying. The pots were irrigated daily with tap water carefully to prevent loss of spore suspension through leaching or excessive drying.

Plant material

A disease free nursery of most susceptible citrus rootstocks *i.e.* Rough Lemon, was purchased from Citrus Nursery Sanitation Laboratory, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan, grown in sterilized soil and maintained in greenhouse. A total of 80 plants (18 months old) were taken and kept in net house.

Synergistic effect of the *F. semitectum* and *T. semipenetrans* was studied to prove that a synergistic relation exists between these two pathogens. Experiment was conducted in greenhouse under completely randomized design (CRD). Local rootstocks of sour orange (*C. jambhiri Lush*) were grown in sterilized soil (72% sand, 17% silt, 8% clay). were used. At the age of 18 months they were inoculated with citrus nematodes alone, *F. semitectum* alone and in combination of *F. semitectum* + *T. semipenetrans.* A treatment of healthy plants served as control. There were four treatments and each replicated fifteen times. All the treatments were arranged on bench in glass house with RH-80-90% at 25±2°C in CRD for three months.

Data recording

Experiment was harvested after three months. Data was recorded on fresh weight of shoot, fresh weight of root, shoot length, root length, number of leaves, number of females/gram of roots, number of J2/g of roots and number of J2/100 ml of soil. Experiment was repeated again to confirm the results. Results were subjected to analyze by using M-Stat version 8.1 and treatment means were compared by Least Significant Difference Test at 5% significance level. The association of inoculated pathogens was confirmed by reisolation.

RESULTS

Synergistic effect of the *F. semitectum* and *T. semipenetrans* was studied to prove that a synergistic relation exists between these two pathogens. Experiment was harvested after three months.

Quantity of leaves

The effect of fungi and nematode on quantity of leaves varied significantly (P = 0.05) in individual and combined treatment (Table I). Minimum of leaves were observed in treatment consisting of both pathogens (*Fusarium semitectum* + *Tylenchulus semipenetrans*) together (58 leaves), followed by treatments consisting *T. semipenetrans* (90 leaves) and *F. semitectum* (145 leaves) alone respectively. Similarly 61.33% reduction in number of leaves was observed in treatment consisting of both pathogens together followed by the consisting *T. semipenetrans* (40%) and *F. semitectum* (3.33%) (Fig. 1). Number of leaves was significantly higher in healthy plants (150 leaves) and was significantly different from other treatments.

Root length (cm)

Effect of fungi and nematode individual and combined treatment was evaluated for their effect on root length. Results showed that the maximum root length was observed in healthy plants (32.01 cm) (Table I). It was followed by treatments consisting of fungi (29.80 cm) and nematode (26.00 cm) which were applied separately. But the effect of fungi on root length was not significantly different from healthy plants. Minimum root length was observed in treatment consisting of both pathogens together (19.30 cm) with reduction percentage of 39.7% (Fig. 1) which was significantly different from the other treatments.

Shoot length (cm)

The maximum shoot length was observed in healthy plants (63.00 cm). It was followed by treatments consisting of fungi (58.00 cm) and nematode alone (49.00 cm) having reduction percentage of 7.93% and 22.22% (Fig. 1). Minimum shoot length was observed in treatment containing both the pathogens together (43.03 cm) which was significantly different from the other treatments. Maximum reduction in shoot length (31.74%) was recorded in treatment containing both the pathogens (Fig. 1).

Table I.- Growth response of citrus plants to *T. semipenetrans* and *F. semitectum*, administered alone and in combination.

Plant growth parameters	Treatments			
	T. semipenetrans	*F. semitectum*	*F. semitectum + T. semipenetrans*	Healthy plants
No. of leaves	90.00b	145.0a	58.00c	150.0a
Root length (cm)	26.00b	29.80a	19.30c	32.01a
Shoot length (cm)	49.00c	58.00b	43.03d	63.00a
Fresh shoot weight (g)	19.61c	22.80b	16.14d	24.91a
Fresh root weight (g)	6.81b	8.65a	5.21c	9.01a
Stem diameter (cm)	1.455c	1.829b	1.025d	1.96a
Feeder roots population (no.)	1394.0c	1488.0b	789.0d	1581.0a

Data are mean of fifteen replicates. Means within a column sharing the same letter are not significantly different from each other at $P = 0.05$ according to LSD Test.

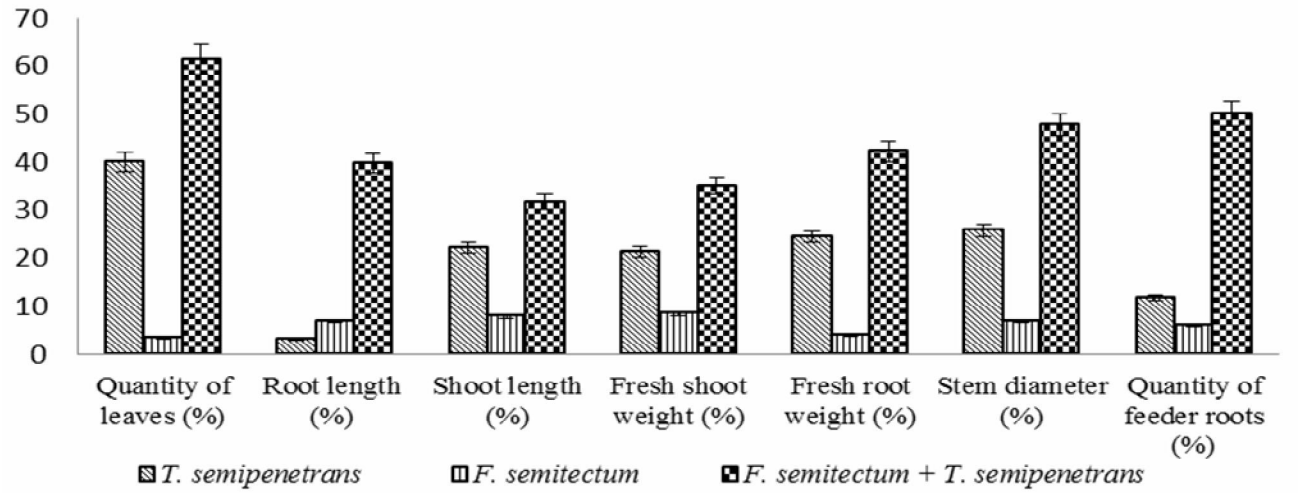

Fig. 1. Percent increase/decrease over control in plant growth parameters of citrus rootstock as influenced by infection with *F. semitectum* and *T. semipenetrans* alone or in combination under greenhouse conditions.

Fresh shoot weight (g)

Maximum reduction in fresh shoot weight (35.2%) was recorded in treatment containing *F. semitectum* and *T. semipenetrans* together followed by *T. semipenetrans* (21.27%) and *F. semitectum* (8.5%) (Fig. 1).

Similarly, from Table I it is clear that minimum fresh shoots weight was observed in the combined treatment consisting of both pathogens *F. semitectum* + *T. semipenetrans* (16.14 g) followed by treatment consisting of *T. semipenetrans* only (19.61 g) and *F. semitectum* (22.80 g). Fresh shoots weight was significantly higher in healthy plants (24.91 g) and was significantly different from other treatments.

Fresh root weight (g)

Minimum fresh roots weight was observed in the combined treatment of *F. semitectum* + *T. semipenetrans* (5.21 g) followed by treatment consisting of *T. semipenetrans* (6.81 g) and *F. semitectum* (8.65 g) respectively. But the effect of fungi on fresh roots weight was not significantly different from healthy plants. Fresh roots weight was significantly higher in healthy plants (24.91 g) and was significantly different from other treatments (Table I). Similarly 42.21% reduction in number of leaves was observed in treatment consisting of both pathogens together followed by the consisting *T. semipenetrans* (24.48%) and *F. semitectum* (4%) (Fig. 1).

Stem diameter (cm)

Maximum stem diameter was observed in healthy plants (1.963 cm). It was followed by treatments consisting of fungi only (1.829 cm) and treatment consisting of nematode only (1.455 cm) showing reduction percentage of 6.82% and 25.87% respectively (Table I, Fig. 1). Maximum reduction in stem diameter was found in treatment containing both pathogens *i.e. F. semitectum* + *T. semipenetrans* (47.78%). Minimum stem diameter was observed in treatment consisting of both pathogens *F. semitectum* + *T. semipenetrans* (1.025 cm) which was significantly different from the other treatments (Table I, Fig. 1).

Quantity of feeder roots (%)

Maximum reduction in feeder roots (50.09%) was recorded in treatment containing *F. semitectum* and *T. semipenetrans* together followed by *T. semipenetrans* (11.82%) and *F. semitectum* (5.88%) (Fig. 1). Similarly, Minimum number of feeder roots was observed in the combined treatment of both pathogens *F. semitectum* + *T. semipenetrans* (789.00) followed by treatment consisting of *T. semipenetrans* (1,394) and *F. semitectum* (1,488) respectively. Number of feeder roots was significantly higher in healthy plants (1,581) and was significantly different from other treatments (Table I, Fig. 1).

Population of attached females

Maximum population of attached females (females/gram roots was observed in treatment consisting of *T. semipenetrans* (589 females/g roots) with 60.22% increase followed by treatment consisting of *T. semipenetrans* + *F. semitectum* together (39.77%) (Table II, Fig. 2). Treatments containing *F. semitectum* and healthy plants were not observed to have any female because they were not inoculated with inoculum of *T. semipenetrans*.

Population of hatched juveniles from roots

Maximum population of hatched juveniles (J_2/gram roots) was observed in treatment consisting of *T. semipenetrans* (8,732 J_2/g roots) (Table II). Treatments containing *F. semitectum* and healthy plants were not observed to have any juvenile because they were not inoculated with inoculum of *T. semipenetrans*. Similarly percent increase in number of J2 per gram roots was recorded in treatment consisting of *T. semipenetrans* (59.97%) followed by treatment containing *T. semipenetrans* + *F. semitectum* together (40.02%) (Fig. 2).

Population of hatched juveniles from soil

The maximum population of hatched juveniles (J_2/100 ml soil) was observed in treatment consisting of *T. semipenetrans* only (3,515 J_2/100 ml) with 61.34% increase followed by treatment consisting of *T. semipenetrans* + *F. semitectum* together (38.65%) (Fig. 2). Treatments containing *F. semitectum* and healthy plants were not observed to have any nematode because they were not inoculated with inoculum of *T. semipenetrans*.

DISCUSSION

Fusarium spp. and *T. semipenetrans* are the most serious soilborne pathogens attacking roots of citrus plants (El-Mohamedy, 1998; El-Nouby, 2002). Many scientists had recorded the relationship between the presence of the citrus nematode and the increase in root rot disease on citrus rootstocks (Van Gundy and Tsao, 1963; O'Bannon *et al*., 1967, Labuschagne *et al*., 1989, Combrink *et al*., 1996; El-Mohamedy *et al*., 2005). The present study tested the response of citrus rootstock, *i.e.* sour orange, to infection with *F. semitectum* and *T. semipenetrans* alone or in combination. Percentage of plant growth parameters were significantly decreased when seedlings were inoculated with *F. semitectum* + *T. semipenetrans* to seedlings inoculated with *F. semitectum* or *T. semipenetrans* separately. With the increase in density of *Fusarium* propagules and nematode in rhizospheric soil of all tested rootstocks, the reduction percentage in growth parameters in citrus seedlings indicate that sour orange was considered susceptible to *F. semitectum* and *T. semipenetrans* when introduced to the soil alone or in combination. Similar findings were also demonstrated by other authors (Labuschagne *et al*., 1989; Combrink *et al*., 1996; El-Mohamedy, 1998; Lucas *et al*., 2000; El-Nouby, 2002; El-Mohamedy *et al*., 2005). This tolerance has been credited partly to rootstock vigour, the capacity of rapidly replacing damaged roots and callus tissue formation (Smith *et*

Table II.- Nematode reproduction parameters in citrus plants after inoculation.

Nematode reproduction parameters	Treatments			
	T. semipenetrans	***F. semitectum***	***F. semitectum + T. semipenetrans***	**Healthy plants**
Population of attached females (females/gram roots)*	589.00a	0.00c	389.00b	0.00c
Population of hatched Juveniles (J_2/gram roots)**	8732.00a	0.00c	5828.00b	0.00c
Population of hatched Juveniles (J_2/100 ml soil)**	3515.00a	0.00c	2215.00b	0.00c

Data are mean of fifteen replicates. Means within a column sharing the same letter are not significantly different from each other at $P = 0.05$ according to LSD Test.
*Counted the attacked females after staining with acid fuchsin.
**Isolated by using Whitehead and Hemming tray method and Baerman Funnel method.

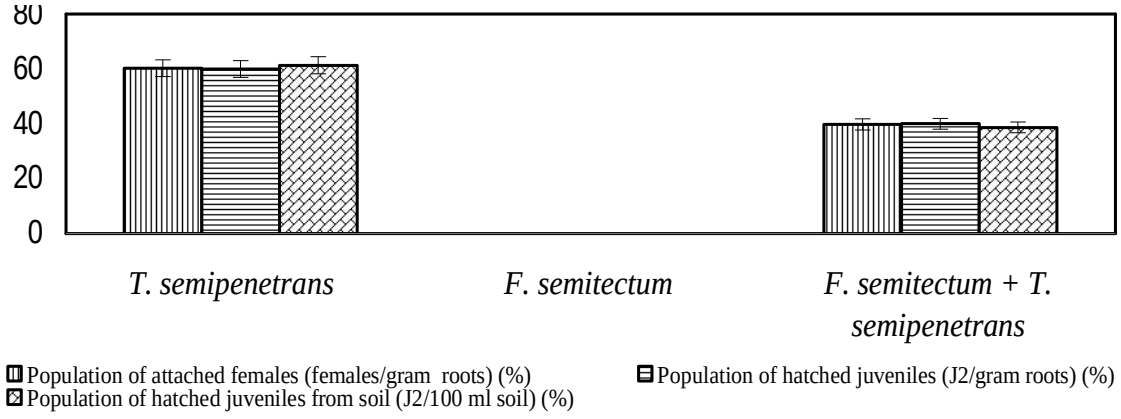

Fig. 2. Percent increase/decrease over control in nematode reproduction parameters of citrus rootstock as influenced by infection with *F. semitectum* and *T. semipenetrans* alone or in combination under greenhouse conditions.

al., 1991; Hough, 1992; Abd El-Zaher and Abo-Elezz., 2002). Moreover, the presence of *T. semipenetrans* in the growth soil could diminish effectiveness of *Fusarium* tolerance rootstocks, which was clearly observed in the present study in all *Fusarium*, nematode and combination treatments

Synergistic effect of the *F. semitectum* and *T. semipenetrans* was studied to prove that a synergistic relation exists between these two pathogens. Data regarding number of leaves, root length, shoot length, fresh shoot weight, fresh root weight, stem diameter and number of feeder roots revealed that all the plant growth variables were recorded lower in treatment consisting of both pathogens (*F. semitectum* + *T. semipenetrans*) together. Number of females per gram roots and number of J2 per gram roots were observed lower in treatment consisting of both pathogens (*F. semitectum* + *T. semipenetrans*) together *i.e.* 589 and 8732, respectively. It may be due to less number of lateral roots produced.

From the results obtained it may be suggested that there was a direct relationship between root growth variables and nematodes population which resulted in lower number of juveniles in soil. The mechanism by which fungus reduce the nematode population may be the root rottening and less number of lateral roots. In the treatment only fungus was applied, all of the plant growth parameters were slightly affected. Fungi significantly also reduced the nematode population in an 8-year-old Valencia orchard (Le Roux *et al.*, 2000).

As *Fusarium* and *T. semipenetrans* both are soilborne so there must be the close association occurring between them (Gene *et al*., 2005). The interaction between these two pathogens has also been demonstrated to be pathogenic to citrus and lead the citrus industry towards decline (Labuschagne *et al.,* 1989). Our results confirmed the findings of different workers who worked on synergism of *Fusarium* and plant parasitic nematodes (Labuschagne *et al*., 1989; Rupe., 1989; Mac-Guidwin and Rouse, 1990; Powelson and Rowe, 1993; Prot, 1993; Melgar *et al.*, 1994; Yang and Rizvi, 1994; Roy *et al*., 1997; Gao *et al*., 2006). During interaction nematode plays the primary role as modifier of the host, making it more susceptible for other pathogens (Pitcher, 1978; Powell, 1979).

This may be due to fact that plant parasitic nematodes search for the roots of host plants during their active phase. Once nematodes have penetrated a root, they cause injury to roots and provide entry of other pathogens into roots. Thus makes the diagnosis of the cause of decline become difficult (Vuuren *et al*., 1991). *Fusarium* is a mild pathogen which gains entry through injured parts thus infection becomes more extensive under adverse environmental condition causing more damage to the plant (Cook, 1969). It develops in vascular tissues by formation of amorphous plugs (Vegas *et al*., 1988). *T. semipenetrans* and the fungus *F. solani*, occurring together or independently, induce plug formation in the xylem but not high zinc concentrations in the wood (Rangel *et al*., 1992). Decline may be due to the effect of naphthazarins (dihydrofusarubin and isomarticin) released by *F. solani* on the cytology of leaves of rough lemon (*Citrus jambhiri*) seedlings (Achor *et al*., 1993; Rensburg *et al.,* 1996). The reduction of carbohydrates in nematode infected citrus roots might have an important impact on the host parasite relationship between *F. solani* and citrus plants (Hamid *et al*., 1985).

Our results strongly disagree with the work of the different researchers who concluded that *F. solani* is nonpathogenic to citrus with inability to develop symptoms during pathogenesis (Graham *et al*., 1985; Dandurand and Menge, 1992). Some workers have suggested that no synergistic relation exists between these two pathogens. The *Fusarium* spp. are predominantly common soil fungi, present in almost all parts of the world as a colonizer of root surfaces or a weak invader of the root cortex of many plants and cause wilts and root rots (Cook, 1969; Armstrong, 1975).

CONCLUSIONS

It is concluded that a synergistic relationship exists between *F. semitectum* and *T. semipenetrans*, which is a potential threat for citrus plants. The present study also leads to suggestion that citrus rootstocks reaction to *Fusarium* spp. and citrus nematode should be a part of an integrated control strategy to alleviate decline of citrus.

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